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Zoospore encystment-based and cabin sequestering methods for isolation and salvaging *Phytophthora* from infested rhizospheric soils

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Abstract *Phytophthora*, one among the most devastating phytopathogenic genus representing the oomycetes inflicts substantial damage to a broad spectrum of economically important horticultural crops worldwide. The rapid dissemination of *Phytophthora* in agro-ecosystems is mediated through bi-flagellated zoospores and their homing response towards the host is profoundly influenced by chemo-electrotactic mechanisms leading to encystment and subsequent colonization. Though different procedures are reported to isolate *Phytophthora* from infested rhizospheric soils, studies addressing zoospore encystment-based method using different baits for isolation and subsequent retrieval of cultures (in case of bacterial contamination) are meagre in *Phytophthora*-black pepper host-pathosystem. In the present study, *Phytophthora* was isolated from infested rhizospheric soils of black pepper using different baits viz., thermocol, impregnated paper discs, leaves of *Albizzia falcataria* and green gram roots. Modified cabin-sequestering methods were subsequently adopted to salvage the cultures in case of bacterial contamination, if any. The zoospore encystment-based method is cost effective and consumes lesser time for isolation of *Phytophthora* from infested rhizospheric soils of black pepper. Further, the two approaches of cabin sequestering method which were found promising in obtaining pure cultures of *Phytophthora* devoid of bacterial contamination, can also be employed to retrieve bacterial contaminated cultures of research significance.

Keywords Black pepper · Cabin sequestering · Bacterial contamination · Encystment · *Phytophthora* · Tactic response

Abbreviations

CA	Carrot Agar
CTAB	Cetyl Trimethyl Ammonium Bromide
BHMPPVR	Benlate Hymexazol Mycostatin Pentachloronitrobenzene Pimaricin Vancomycin Rifamycin
NARH	Nystatin Ampicillin Rifampicin Hymexazol
PVPH	Pimaricin Vancomycin Pentachloronitrobenzene Hymexazol

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PARP	Pimaricin Ampicillin Rifampicin Pentachloronitrobenzene
P_{10}ARP	Pimaricin (10 µg/ml) Ampicillin Rifampicin Pentachloronitrobenzene
P_{10}VP	Pimaricin Vancomycin Pentachloronitrobenzene

Introduction

Phytophthora, the Stramenophilic phytopathogen representing the extremely destructive oomycete cluster is considered as the most devastating and prominent genus pan globe. This genus currently encompasses more than 180 species, with more predicted to be discovered as new natural habitats are explored (Scott et al., 2019). Considering the irrefutable economic significance, research endeavours are currently focussed on developing reliable as well as robust tools to unravel information on pathogen diversity which may further aid in formulating specific management strategies for *Phytophthora* induced diseases in various crops (Assinder, 2004). The global importance of *Phytophthora* species as plant pathogens in agriculture, horticulture, and natural ecosystems fundamentally necessitates isolation and culturing under in vitro conditions for subsequent investigations. In black pepper, the most devastating *Phytophthora* foot rot caused by *Phytophthora capsici* and *P. tropicalis* (Jeevalatha et al., 2021; Suseela et al., 2022) infects all the aerial as well as subterranean plant parts, including the stem, spikes, leaves, and roots. Once the parasitic relationship is established with host, the pathogen proliferates and disseminates rapidly, ultimately leading to epiphytotics under field conditions.

The rapid dissemination of *Phytophthora* in nature is chiefly mediated through the asexually produced wall-less, uninucleated, non-proliferating bi-flagellated propagules i.e., the zoospores. The homing response of the motile free-swimming zoospores aided through chemo- and electrotaxes have profound influence in dictating the cascade of events in pathogenesis. Spontaneous convergence and development of large-scale patterns of cell density (autoaggregation) propelled by distinct time-separated unique mechanisms viz., bioconvection and chemotaxis, further strengthens this global pathogen to invade several horticultural crops (Savory et al., 2014). Evolution of tactic responses of zoospore towards host root confer selective advantages to *Phytophthora* species. Tactic responses include directed movement in chemical gradients (chemotaxis), in water currents (rheotaxis), due to gravity (geotaxis), and in electrical fields (electrotaxis). Chemotaxis enables zoospores to target plant roots by swimming towards regions of nutrient exudation, such as the root apex and wound sites. Rheotaxis and geotaxis are advantageous because the zoospore population are more concentrated in the aerobic surface layers of soil. Plant roots generate electrical fields in the rhizosphere as a consequence of electrogenic ion transport systems operational in the root system. The zoospores of *Phytophthora* are anionic in nature and are attracted towards positive charge. However, the electrotactic response of zoospores has received comparatively less attention which might also act synergistically with chemotaxis in facilitating locating the host (Morris & Gow, 1993).

Maintaining pure cultures is a fundamental pre-requisite to undertake basic scientific research wherein bacterial contamination pose a constant threat and necessitates developing simple, reproducible, and cost-effective methods to completely eliminate the contaminants. Bacterial contamination in microbial cultures is a persistent bottleneck in microbiological research, which hinders both establishment and maintenance of pure cultures. The conventional approach to thwart bacterial contamination is amending the culture media with appropriate antibiotics which has several drawbacks. It is reported that, selective media such as PVPH (Pimaricin, Vancomycin, Pentachloronitrobenzene, Hymexazol) (Tsao & Guy, 1977), PARP (Kannwisher & Mitchell, 1978) and NARH (Nystatin, Ampicillin, Rifampicin, Hymexazol) (Suchana et al., 2020) can be used for isolation and culturing *Phytophthora* species however, the preservation and maintenance of microbial cultures are troublesome task due to the frequent contamination by bacteria and other fungal contaminants. A cocktail of antibiotics-fungicide containing ampicillin, cefotaxime and carbendazim for the removal of fungal and bacterial contamination in green algal cultures (Kan & Pan, 2010) and nano silver for removal of bacterial contamination in valerian tissue culture (Abdi et al., 2008) are reported. Several handy protocols based on non-antibiotic physical methods and selective media have been reported by earlier researchers to eliminate the contaminants and to obtain pure cultures (Brown,

1924; Machacek, 1934; Sleeth, 1945; Ko et al., 2001; Raper, 1937; Harrower, 1989; Cother & Priest, 2009). In order to prevent bacterial contamination in fungal cultures, Shi et al. (2019) developed an effective and easy method referred as cabin sequestering which was proved to be dominant over the antibiotic treatments and other handy techniques reported earlier.

Early detection of the oversummering plant pathogens either in the infected moribund/dead host tissues or in the infested soil continuum is highly imperative to devise prophylactic plant protection measures during the ensuing season. Furthermore, early detection also provides first-hand information on presence or absence of the pathogen before establishing a new crop. Recently, robust technologies viz., high-throughput sequencing and metabarcoding of eDNA have been employed to detect microbes from diverse environmental niches. Though proved to be promising, these technologies, does not provide vital information on whether the microbe is viable or potentially pathogenic. Several simple and handy conventional methods have been devised to isolate *Phytophthora* from infested sources including apple fruit (Campbell, 1949), cocoa pods (Orellana, 1954), lupin seedlings (Pratt & Heather, 1972), taro roots (Satyprasad & Ramaro, 1980) and leaves of *Albizzia falcataria* (Anandaraj & Sarma, 1990). However, the major lacunae of employing *A. falcataria* leaves as baiting substrate to isolate *P. capsici* infecting black pepper are delay in the infection process and subsequent time lag in the symptom development as well as contamination due to soil-dwelling facultative and saprophytic microbes. Several specific media supplemented with different combination of antibiotics are used for the isolation of *Phytophthora* from infested sources. However, recurrent use of antibiotics leads to evolution of resistant bacterial contaminants, which challenges the purification procedure. Earlier developed purification techniques require expertise and the possibility of recontamination warrants the refinement of existing techniques. Pertinent information on zoospore encystment-based method for isolation and subsequent retrieval (in case of bacterial contamination) of cultures are scanty with respect to *Phytophthora*-black pepper host-pathosystem. Hence, in the present study attempts were made to isolate *Phytophthora* based on the electrotactic behaviour and encystment of zoospores from infested rhizospheric soils of black pepper and also assess the utility of earlier reported cabin sequestering method with two different approaches intended for refinement, to avoid the possibility of recontamination and to salvage bacterial contaminated *Phytophthora* cultures.

Materials and methods

Phytophthora isolate: *Morpho*-molecular characterization and pathogenicity

The black pepper isolate of *Phytophthora* was obtained from National Repository of *Phytophthora*, ICAR-Indian Institute of Spices Research, Kozhikode, Kerala, India. The mycelial plugs which were stored in vials containing sterile distilled water were transferred onto carrot agar (CA) medium for revival and pure cultures thus obtained were cultured on CA medium. For morphological characterization, the mycelial plugs of 5 mm dimension were transferred onto CA medium and incubated at 24 ± 1 °C. The micro-morphological features were examined under compound research microscope (LEICA DM 5000B), dimensions recorded and photomicrographs were documented. Further, identity of the isolate was confirmed employing species-specific primers viz., PC-Y-FP (5'-AAGCAACCAAAGTTCAAGACGTTT-3'), PT-Y-FP (5'-GATTGTAAGCACTTGTTATCC ATC-3') and COM-Y-RP2 (5'-GCAGGCGTATCT GAAATTTGC-3') (Jeevalatha et al., 2021). Briefly, the DNA was isolated adopting the standard protocol described by Sheji et al. (2009). The polymerase chain reaction (PCR) was performed in Gradient Thermal Cycler IG-96GEP with initial denaturation at 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 1 min, primer annealing at 60ºC for 1 min, extension at 72ºC for 1 min and a final extension at 72ºC for 10 min. Twenty µl PCR reaction mixture was prepared with 15.2 µl nuclease-free water, 2 µl of Taq buffer, 0.5 µl dNTP mix, 0.5 µl of forward and reverse primer, 0.3 µl of Taq polymerase and 1 µl of genomic DNA (approximately 100 ng). The PCR amplified products were resolved on 1.5 X agarose gel containing ethidium bromide for 1 h and 30 min at constant 85 V and documented using GeneSys software. The PCR product was purified using NucleoSpin® Gel and PCR Clean-up Column kit and sequenced.

Pathogenicity of the isolate was proved on susceptible black pepper variety Sreekara. For this, rooted cuttings raised through serpentine method were transplanted to polythene bags (21 cm × 15 cm dimension) and plants at 4–5 leaf stage were used for inoculation. The isolate was cultured on CA medium and incubated for 72 h and the mycelial plugs (3 mm dimension) were derived from the advanced margins of the actively growing 72 h old cultures. The sporulating mycelial plugs were placed on the abaxial surface of fully unfurled second leaf (without injury) over which sterile moistened cotton was placed to maintain adequate moisture to facilitate the infection process. The inoculated plants were maintained for 72 h at an ambient temperature of 24–25 °C with relative humidity of 80–90% (ambient weather conditions) and regularly observed for the development of symptoms (Prakash et al., 2019).

Preparation of zoospore suspension

The zoospore suspension was prepared by placing plugs of CA medium with 4 days-old culture in 2 mM sodium phosphate buffer (pH 7.2) and maintained at ambient room temperature (27 ± 2 °C) for 48 h under continuous illumination. The suspension was cold shocked at 4 °C for 30 min to facilitate the release of zoospores. The suspension was then filtered through Whatman No. 54 filter paper in order to obtain a pure suspension of zoospores (van West et al., 2002).

Zoospore-tactic assay: Analyzing electrotactic behaviour of zoospores

The effect of electrical fields on zoospore movement was studied using a fabricated electrotaxis chamber (Morris and Gow, 1993) with minor modifications. The chamber consisted of a glass slide with a central channel formed between two sections of glass. Copper wire electrodes were glued to both ends of the channel and molten agarose gel (1% w/v) was poured over the electrodes and both ends of the channel to a central well with dimensions of 4.5 × 4.5 cm and 0.2 cm depth (Fig. 1). An aliqout 100 µl of zoospore suspension was placed in the central chamber and overlaid with a coverslip prior to the application of electrical field across the chamber. The zoospore suspension of *Phytophthora* was placed in the field of 500 mV cm^{-1} (van West et al., 2002). The movement and aggregation of zoospores inluenced by the electric field were observed under a compound microscope (LEICA DM 5000B) at periodic intervals and documented.

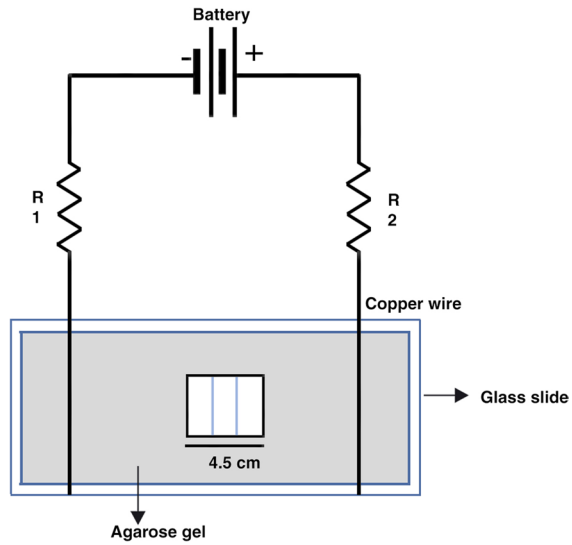

Fig. 1 Diagrammatic representation of electrotaxis chamber

Standardization of isolation procedures

Preparation and storage of soil samples

For initial standardization, soil samples were collected from the rhizosphere of black pepper grown under field conditions (disease-free). The samples were consolidated, mixed thoroughly, autoclaved at 121 °C at 15 lbs for 20 min, sieved to obtain < 2 mm fractions, mixed thoroughly again and stored at ambient temperature (25 ± 2 °C) in plastic bags under laboratory condition. The rhizospheric soil samples were also collected from two locations (Kozhikode, Kerala and Kodagu, Karnataka, India) representing foot rot affected black pepper plots. For activation of perennating structures, the protocol described by Schmitthenner and Bhat (1994) was employed with modifications. Briefly, the soil was flooded for 1 h, excess water was drained and air dried. The soil was then stored in polyethylene bags to prevent further drying and incubated at room temperature for a period of one week from flooding.

Preparation of baits

(i) Plant species:
For preparing live baits, *Albizzia falcataria* and green gram were selected considering the easy availability or easiness in production of large number seedlings within a short period.

(a) The leaves of *Albizzia falcataria* (previously reported by Anandaraj & Sarma, 1990) collected from live trees were used.
(b) In case of green gram, the seeds were subjected to pre-soaking for 12 h (overnight) in water and placed over moistened filter paper in a Petri dish. The seeds were moistened periodically by spraying sterile distilled water to maintain adequate moisture till germination and further until the experiments were executed.

(ii) Inert substrates: To effect the isolation of *Phytophthora* based on zoospore encystment, inert substrates viz., thermocol (0.5 cm diameter) and impregnated paper discs (0.5 cm × 0.5 cm) were used.

For preparation of impregnated paper discs, Whatman no.3 filter paper was used. The stock solution of fungicide-antibiotic mixture [propiconazole (0.1%), rifampicin and chloramphenicol each at 10 ppm concentration] was prepared and used for disc preparation. The fungicide and antibiotics were selected considering their broad-spectrum activity against common soil dwelling fungal as well as bacterial contaminants. The autoclaved discs of 0.5 cm X 0.5 cm dimension were placed in Petri dishes approximately 5 mm apart and 20 µl of the fungicide-antibiotic solution was pipetted on each disc. The impregnated paper discs were subsequently dried in an incubator at 37 °C for 4 h and stored at -20 °C until used.

Preparation of soil-zoospore suspension

Twenty five grams of the test soil samples (after the incubation period) was apportioned into a conical flask (250 ml) and 100 ml of sterile distilled water was added, mixed thoroughly to obtain uniform soil–water suspension and allowed to settle so that 2–3 mm of free water stood above the suspension when the particles settled (Anandaraj & Sarma, 1990). The zoospore-tactic and encystment-based isolation was performed in Petri dishes in which the sterile soil artificially amended with zoospores and naturally infested rhizospheric soil samples were used. In order to confirm the presence of common soil-borne fungal and bacterial contaminants associated with foot rot affected black pepper rhizosphere, the soil samples were subjected to serial dilution and the population was enumerated.

Incubation and isolation

The leaves of *Albizzia falcataria* and roots of green gram seedlings with distinct root hair and root cap region of 3 to 4 cm length without any wounds and the inert materials (thermocol and impregnated paper discs) were immersed in Petri dishes with soil-zoospore suspension containing bacterial and fungal contaminants (isolated from the rhizospheric soil) and incubated at ambient room temperature (28 ± 2 °C) for 2 h. Besides the roots of green gram, black pepper roots were also employed to analyze the zoospore-tactic behaviour which was documented using compound microspore at regular intervals.

After incubation period, the baits were transferred separately to modified carrot agar medium (medium amended with propiconazole (0.1%), rifampicin and chloramphenicol, each at a concentration of 10 ppm) and CA medium (without fungicide-antibiotics) in triplicates and maintained under ambient room temperature.

Modified cabin sequestering methods

Characterization of bacterial contaminants

Initially, the bacterial contaminants of *Phytophthora* cultures were pure cultured and identified at genus level. The genomic DNA of bacterial cultures was extracted using CTAB and PCR was performed using the primer pairs 27F (5'-AGA GTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') (de Lillo et al., 2006). The temperature profiles were as follows: an initial pre-heat for 2 min at 94ºC, followed by 35 cycles of denaturation at 94ºC for 1 min, annealing at 52 ºC for 1 min and extension at 72

ºC for 1 min. Twenty µl PCR reaction mixture was prepared with 15.2 µl nuclease free water, 2 µl of Taq buffer, 0.5 µl dNTP mix, 0.5 µl of forward and reverse primer, 0.3 µl of Taq polymerase and 1 µl of genomic DNA. PCR amplified products were electrophoretically separated on 1% agarose gel containing ethidium bromide for 60 min at constant 85 v and documented using GeneSys software. PCR product was purified by using NucleoSpin® Gel and PCR Clean-up Column kit and sequenced.

Modified cabin sequestering methods

For initial standardization, the CA medium (without fungicide-antibiotics) overlaid with identified isolated bacterial cultures were maintained at 37 °C overnight and 5 mm mycelial plugs of *Phytophthora* were inoculated onto the Petri dishes containing bacterial growth. Then attempts were made to salvage bacterial contaminated cultures of *Phytophthora* adopting the method developed by Shi et al. (2019) modified with two approaches. In the first approach consisting of two methods, mycelial discs (3 mm diameter) derived from contaminated *Phytophthora* cultures were positioned with the mycelial growth facing upwards and downwards into a cabin (1 cm diameter) excavated on CA medium separately over which a sterile coverslip (2 cm × 2 cm) was placed thereby creating a completely sealed cabin. In the second approach encompassing two methods, mycelial discs with the hyphae facing upwards as well as downwards were placed on the sterile cover slip which was subsequently positioned over the cabin. The hyphal initials which emerged along the periphery of cover slips 72 h post incubation at ambient temperature with no bacterial contamination were transferred to CA medium to obtain pure cultures. After initial standardization, the bacteria contaminated *Phytophthora* cultures obtained through zoospore encystment-based method were transferred to CA medium for validation. The comparative efficacy of each modified cabin sequestering methods was also analyzed by recording the time period required for the mycelium to come in contact with the culture medium and its subsequent growth based on microscopic observation (LEICA DM 5000B). Further, purity of the cultures (free of bacteria) was confirmed using PCR assay with the universal primers i.e., 27 F and 1492 R.

Results

Characterization of *Phytophthora* isolate: Morphological, molecular and pathogenicity

The colony of *Phytophthora* isolate exhibited cottony morphology with concentric zonations on CA medium and produced papillate ovoid deciduous sporangium (43 × 20 µm) with long pedicel (161 µm). The pathogenicity of *Phytophthora* isolate was proved through *in planta* assay in which characteristic blackish water-soaked lesion was developed on black pepper leaves four days post inoculation. PCR assay with PC-Y-FP, PT-Y-FP and COM-Y-RP2 primers generated the expected 301 bp amplicon confirming identity of the pathogen at species level as *Phytophthora tropicalis* (Fig. 2).

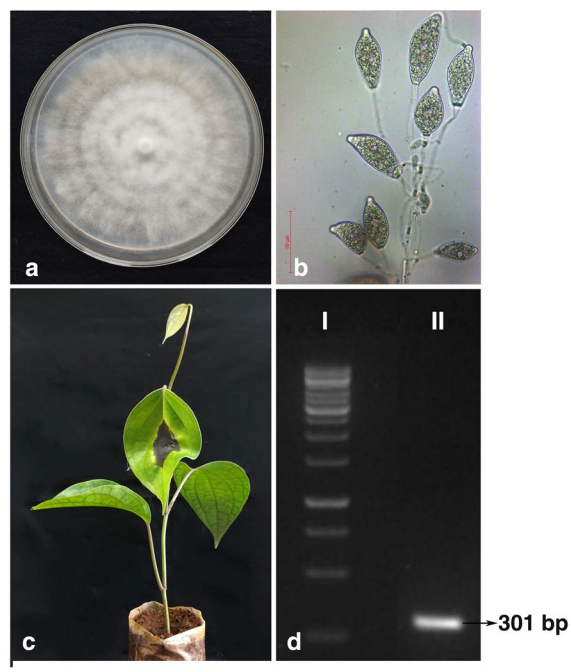

Fig. 2 Characterization of *Phytophthora* isolate: (a) Colony (b) Sporangium (c) Pathogenicity (d) Molecular identification: lane I-1 kb ladder, lane II-*Phytophthora tropicalis*

Analyzing electrotactic behaviour of zoospores

The electrotactic behaviour of zoospores was analyzed employing an electrotaxis chamber. Microscopic observations revealed that, the zoospores aggregated mainly at the anode indicating their anodotactic nature.

Isolation of *Phytophthora* from infested rhizospheric soil

For isolation from the infested rhizospheric soils of black pepper, the roots of green gram, leaves of *Albizzia falcataria*, thermocol and impregnated paper discs were immersed in the soil suspension for 2 h (Fig. 3 a) and later placed on modified carrot agar medium and carrot agar medium (without fungicide-antibiotics). At an interval of 30 min, the green gram roots were examined under compound microscope and found that the zoospores mainly aggregated on the root apical region (Fig. 3 b) compared to root elongation region. After 1 h of incubation, the zoospores encysted on the root surface and germination commenced after 2 h. On modified CA medium, the mycelial growth was observed from the baits 3 days after incubation under ambient conditions (Fig. 4.1). However, the baits inoculated on CA medium (not amended with fungicide-antibiotics) were found to be contaminated with bacteria as well as fungi (Fig. 4.2). The method was subsequently validated with the soil samples collected from two diverse geographical regions representing Karnataka and Kerala experiencing distinct agro-climatic conditions,

Though the modified carrot agar medium amended with fungicide-antibiotics could eliminate most of the soil-borne contaminants, in few instances bacterial contaminants were found associated with the cultures of *Phytopthora* isolated from rhizospheric soils (Fig. 5). Hence, the cabin sequestering method with different approaches were designed to eliminate and salvage the *Phytophthora* cultures from bacterial contaminants.

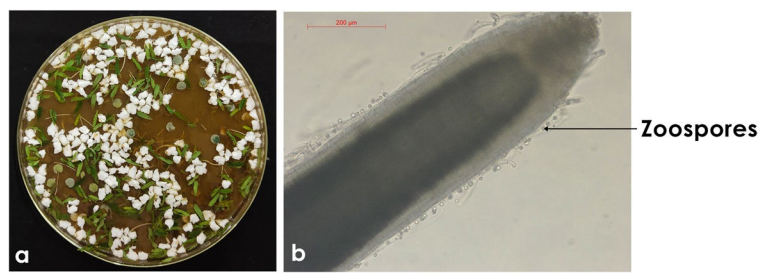

Fig. 3 (a) Soil zoospore suspension with different baits (b) zoospore aggregates on green gram root tip

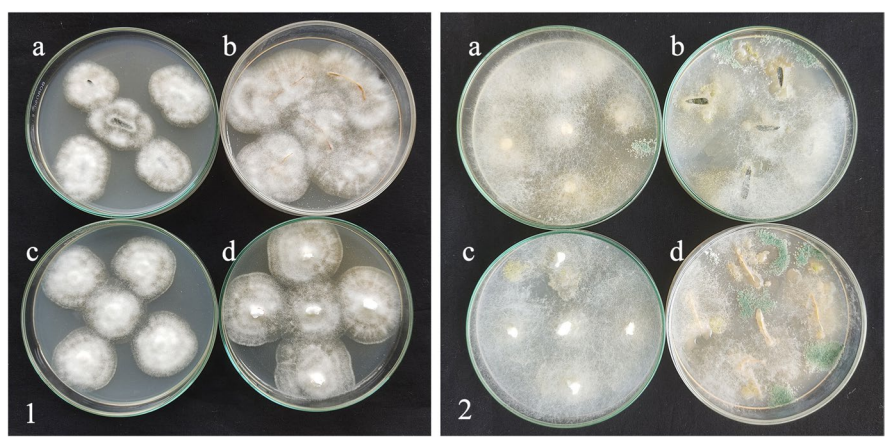

Fig. 4 (1) Baits after incubation on modified carrot agar medium and (2) non-amended carrot agar medium (a) *Albizzia* leaves (b) green gram roots (c) impregnated paper discs (d) thermocol

Fig. 5 *Phytophthora* cultures with bacterial contamination

Salvaging *Phytophthora* cultures from bacterial contaminants: Modified cabin sequestering methods

Characterization of bacterial contaminants

In the initial standardization attempt, the bacteria isolated from contaminated *Phytophthora* cultures were identified based on colony characteristics and molecular assay. The colonies of *Staphylococcus* appeared white coloured whereas the colonies of *Chryseobacterium* were orange to yellow. PCR assay with 27F and 1492R primers generated the expected 1500 bp amplicon which was subsequently sequenced and identified at genus level based on BLAST analysis. In BLAST analysis, the *Staphylococcus* sp. was 100% similar to *Staphylococcus* sp. strain GERBI (MH473522) and the *Chryseobacterium* sp. had 99.68% similarity to *Chryseobacterium* sp. strain ON_mb1 (LC487322) from NCBI.

Modified cabin sequestering methods

The bacterial contaminated mycelial plugs of *Phytophthora* culture were inoculated in four different ways as mentioned earlier. After 72 h of incubation at ambient temperature the hyphae grew along edges of the cover slip and emerged on the surface of the culture medium. Later the mycelial plugs from the Petri dishes were sub-cultured to obtain pure cultures (Fig. 6). The morphology of salvaged cultures was similar to that of the original colony morphology of *Phytophthora* culture. The modified cabin sequestering methods revealed that the methods were promising in removing bacterial contamination without the use of antibiotics. Subsequently, validation of the methods was undertaken to salvage the *Phytophthora* cultures isolated from naturally infested rhizospheric soils of black pepper representing different geographical locations.

The *Phytophthora* cultures which were subjected to different modified methods of cabin sequestering were observed microscopically at periodic intervals (12 h to 120 h). The hyphae originating from the contaminated mycelial plug were totally free from bacterial contaminants and the cabin was found free from bacterial growth (Fig. 7).

Molecular confirmation of removal of bacterial contaminants

The pure cultures of *Phytopthora* salvaged through cabin sequestering methods were analyzed using PCR assay with universal bacterial primers (27F and 1492R). No amplicons were generated from the cultures obtained using all the four modified cabin sequestering methods. Whereas, amplicons of approximately 1500 bp were generated in positive control (bacterial DNA) and from the contaminated *Phytophthora* cultures. These results confirmed that the modified cabin sequestering methods completely removed the bacterial contaminants (Fig. 8).

Discussion

The oomycete phytopathogen, *Phytophthora* poses a threat to a diverse array of economically important horticultural crops, including spices (Kennedy & Duncan, 1995; Erwin & Ribeiro, 1996). It is a major pathogen in spices and incite a broad spectrum of destructive diseases in black pepper, cardamom, nutmeg, and vanilla (Biju et al., 2017). Foot rot is the most prevalent and devastating disease caused by *Phytophthora* spp. which has resulted in significant economic loss in black pepper cultivating regions.

Collection, conservation and long-term preservation of the cultures representing different agro-ecological regions, climatic patterns, hosts as well as cropping systems are vital in undertaking investigations under laboratory conditions to understand the evolutionary biology, analyzing population diversity,

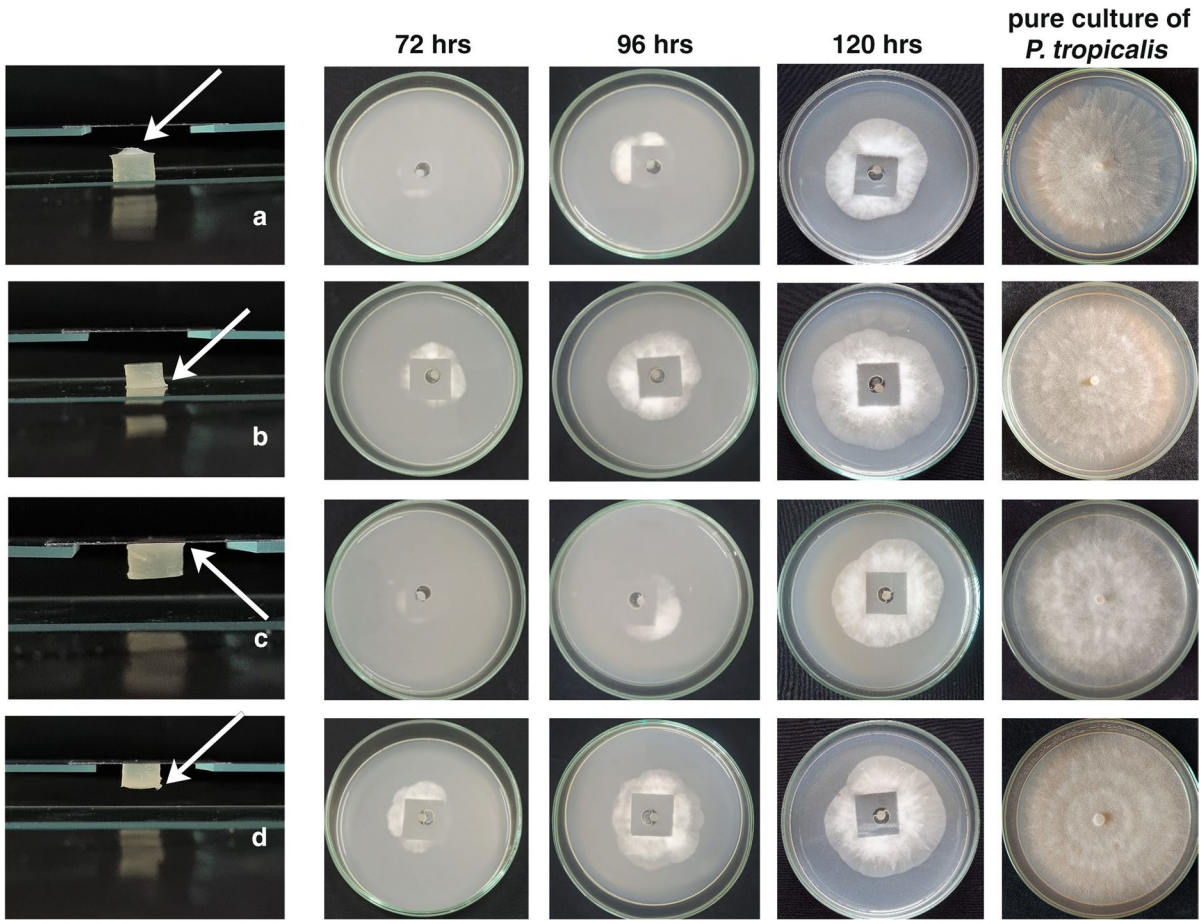

Fig. 6 (a) Method 1: mycelial plug facing towards the coverslip (b) method 2: mycelial plug facing downwards (c) method 3: mycelial plug attached to coverslip and facing upwards (d) method 4: mycelial plug attached to coverslip and facing downwards. Mycelial growth at 72 h, 96 h and 120 h of incubation, respectively and pure cultures of *P. tropicalis* obtained from each method

delineate host–pathogen interaction and generating preliminary information on the efficacious molecules against the pathogen subsequently to formulate management strategies. Long-term preservation of pure microbial cultures without losing its unique inherent genotypic characteristics and periodical retrieval without any contamination encompasses the fundamental and critical phases in microbiological research.

Zoospores are asexual biflagellate spores lacking cell wall formed within the sporangium. The movement of zoospores is principally governed by several external stimuli which leads to homing, docking and encystment on the host surface (Khew & Zentmyer, 1973). Khew and Zentmyer (1973) investigated electrotaxis behaviour in seven species of *Phytophthora* (*P. cactorum*, *P. capsici*, *P. cinnamomi*, *P. citrophthora*, *P. megasperma* var. *sojae*, *P. palmivora* and *P. parasitica*) and stated that zoospores exhibited an active, directed attraction towards the anode, followed by encystment and germination, indicating its anionic nature. Morris and Gow (1993) reported the electronegative nature of anterior flagellum of *P. palmivora* zoospore by validating the anodotactic behaviour of zoospores under applied electric field. The present study investigated the electrotactic behaviour of zoospores of *P. tropicalis* and found that, they exhibited an anionic nature under electric field. van West et al. (2002) examined the movement of *Phytophthora palmivora* zoospores towards the

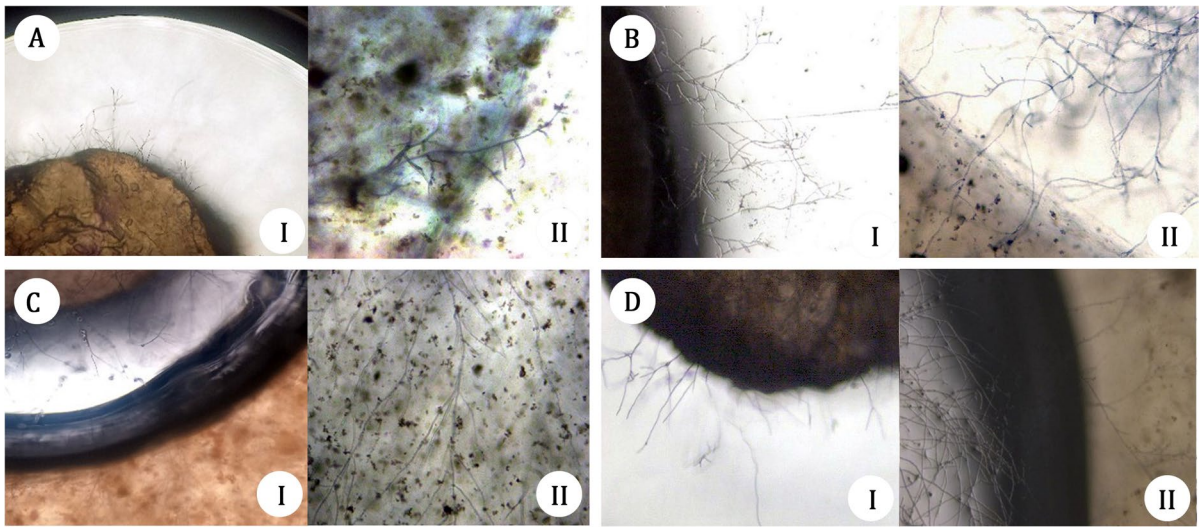

Fig. 7 Photomicrographs depicting mycelial growth in different methods (24 h after incubation): (A) method 1, (B) method 2, (C) method 3, (D) method 4 (I = hyphal initials originating from mycelial disc, II = hyphal growth in the medium)

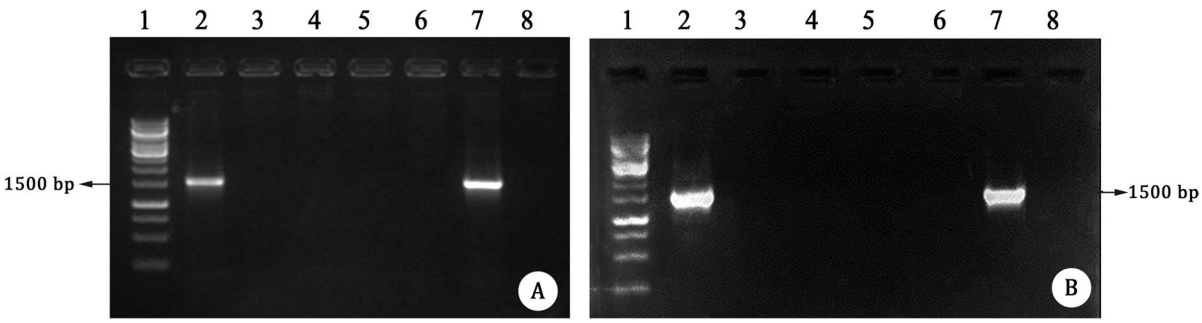

Fig. 8 Lane 1- kb ladder, lane 2- Contaminated culture, lane 3 to 6 method 1 to method 4, respectively, lane 7 -pure bacterial DNA, lane 8- water control. (A) *Phytophthora* culture contaminated with *Staphylococcus* (B) *Phytophthora* culture contaminated with *Chryseobacterium*

electric field produced by rye grass root. They found a correlation between the accumulation of zoospores and electric field generated around the root system, i.e., the anodotactic root apex of rye grass attracted negatively charged zoospores towards the root apical region.

According to earlier reports, *Phytophthora* species were isolated from soil using different live baits, including apple, cocoa pods (Lee & Varghese, 1974), lemon (Tsao, 1960), alfalfa (Marks & Mitchell, 1970) and avocado fruit as well as seedlings (Zentmyer, 1980). The leaves of *Loranthus parasiticus*, leaves and young fruits of *Terminalia catappa* were used as baits for isolating *P. palmivora* infecting coconut (Sharadraj & Chandramohanan 2016). Different kinds of baits have been used to isolate *Phytophthora* irrespective of their host and source of inoculum. Hence, in the present study the roots of green gram, a leguminous species was used to understand the electric charge-based movement of zoospores towards a non-host plant and for isolation. It is reported that in soil continuum, the free swimming zoospores are attracted towards both host as well as non-host roots as influenced by chemo or electro gradients (Hardham, 2001). The isolation of *Phytophthora* using *Albizzia* leaves were earlier reported by Anandaraj and Sarma (1990), in which leaves were allowed to float on the soil suspension until the leaves become

symptomatic from which isolation was performed. Zoosporic microbes are reported to be the early and rapid colonizers of insoluble substrata in freshwater environments. It is reported that, cellulose (as filter paper), chitin and glass fibre were used as baits to isolate *Pythium* species and *Rhizophlyctis rosea* from streams which may be due to passive accumulation of the motile zoospores on these inert substrates (Park & McKee, 1978; Park, 1980). Hence, in the present study inert substrates like paper discs and thermocol were used. The paper discs, though impregnated with fungicide and antibiotics were not effective in inhibiting the growth of both soil-borne fungal and bacterial contaminants. This could be probably due to dilution of the chemical components when immersed in soil–water suspension.

Microbial culture contamination is by far the most persistent obstacle encountered in the laboratories with devastating repercussions. Microbial contamination is invariably caused by avoidable technical or handling errors and misguided processes (Cother & Priest, 2009). A biological contaminant is considered as a microbe readily noticeable or cryptic/latent, usually encountered in a culture system that is undesirable and causing adverse effects on the system. The rapid multiplication of the contaminants is generally favoured by relatively undefended environment thereby attaining high population densities leading to alterations in the growth pattern as well as intrinsic characteristics of the culture. The cryptic biological contaminants may persist indefinitely in preserved cultures causing subtle significant modifications in their behaviour (Ko et al., 2001). Brown (1924) described a method for eliminating the contaminants by inverting a segment of agar culture with the contaminated fungus and deriving the inoculum from the lower surface which involved double handling of the contaminated culture. Machacek (1934) developed a handy procedure to eliminate bacterial contamination by placing a small part of contaminated agar culture onto molten and cooled agar, pressing a sterile coverslip onto the agar to seal top of the culture so as to prevent the movement of bacterial contaminant thereby enabling culturing from uncontaminated subsurface hyphal growth. Sleeth (1945) employed a defined nutrient agar with pH 5.0–5.5 to remove bacterial contaminants. Constantinescu (1990) opined that purifying contaminated fungal cultures using agar medium was not novel. Ko et al. (2001) described two methods for the removal of bacteria and mites from contaminated cultures of *Phytophthora*, *Pythium* and *Botryosphaeria* in which a heated blade was employed to remove bottom section of the plastic Petri dish containing the mites or contaminant, scraping the exposed agar and subsequently transferring to fresh agar. Non-antibiotic methods like Raper's rings (Raper, 1937) and van Teighem rings (Harrower, 1989) were also developed based on the principle of growth pattern of filamentous fungi which grow into the agar within the ring and emerge into agar outside, without any bacterial contaminants. Cother and Priest (2009) developed a simple method for removing bacteria from the contaminated cultures of *Ascochyta rabeii, Botryosphaeria dothidea, Botrytis cinerea, Colletotrichum acutatum, Cylindrocarpon* sp., *Eutypa lata, Fusarium* spp., *Guignardia mangiferae, Leptosphaeria maculans, Marssonina rosae, Monilinia fructicola, Phoma caricae-papayae, Phomopsis leptostromiformis, Phyllosticta telopeae, Phytophthora cinnamomi, P. fallax, P. medicaginis, Pleiochaeta setosa, Pythium* spp., *Sarocladium attenuatum, Sclerotinia minor, S. sclerotiorum, Sphaceloma* sp., *Trichoderma* spp. and *Verticillium* spp.

Antibiotics, considered as the most effective strategy to prevent and subjugate commonly occurring bacterial contaminants nevertheless can be risky when overused or used incorrectly. Moreover, when antibiotics are routinely used, resistant strains may develop which may be slow growing and causing low level infections difficult to detect by direct visualization (Fogh et al., 1971). Till date, a variety of antibiotic-amended media have been reported to avoid bacterial and fungal contamination like P_{10}VP (Tsao & Ocana, 1969), PVPH (Tsao & Guy, 1977), BHMPPVR (Bisht & Nene, 1988), hymexazol amended medium (Masago et al., 1977) and P_{10}ARP (Kannwisher & Mitchell, 1978). Sarker et al. (2020) reported NARH medium comprising nystatin, rifampicin, ampicillin and hymexazol and another medium with similar composition amended with chloramphenicol. Sharadraj and Chandramohanan (2016) developed an isolation method for *P. palmivora* comprising carbendazim (125 ppm) and rifampicin (200 ppm). Because of the risk of recontamination, the impact of antibiotics on microbial cultures and cost, these approaches developed for preventing microbial contamination are often insufficient. In the present study, the carrot agar medium was amended with rifampicin and

chloramphenicol considering its inhibitory activity against both Gram-positive and Gram-negative bacteria. Propiconazole, a triazole fungicide was selected based on its broad spectrum of activity against a wide array of fungi.

Different protocols based on physical and chemical strategies have been reported by earlier researchers to annihilate bacterial contamination in cultures of phytopathogens as well as in cell and tissue culture systems (Brown, 1924; Machacek, 1934; Raper, 1937; Sleeth, 1945; Kannwisher & Mitchell, 1978, Harrower, 1989; Ko et al., 2001; Abdi et al., 2008; Cother & Priest, 2009; Kan & Pan, 2010; Shi et al., 2019; Suchana et al., 2020). However, some of these methods were reported to have certain limitations at various stages of execution and warrants development of a method that is less time consuming, technically feasible and economically viable to curtail bacterial contamination under laboratory conditions especially associated with preserved cultures. Recently, Shi et al. (2019) developed an effective and simple protocol, the cabin sequestering method to retrieve bacterial contaminated cultures of *Fusarium graminearum*, *Colletotrichum gloeosporioides*, *Rhizoctonia solani* and *Botryosphaeria rhodina*, the phytopathogens causing head blight of wheat, anthracnose of grapes, sheath blight of rice and canker in grapes, respectively.

In the present study, two common bacterial contaminants associated with *Phytophthora* cultures were identified, as *Staphylococcus* and *Chryseobacterium.* Staphylococci are Gram-positive bacteria about 0.5–1.0 µm in diameter and grow in clusters, pairs and occasionally in short chains. Staphylococci are non-motile, non-spore forming facultatively anaerobic bacteria representing the family Staphylococcaceae (Masalha et al., 2001). Whereas, *Chryseobacterium* is chemo-organotrophic, rod-shaped Gram-negative bacterium which forms typical yellow-orange coloured colonies due to the production of flexirubin-type pigment. One of the species under this genus (*C. wanjuense*) has also been reported as a biocontrol agent against *P.capsici* infecting pepper (Kim et al., 2012). The four adapted versions of the cabin sequestering method effectively prevented bacterial movement from the contaminated plug to the medium due to increased cabin size. The results from the present study revealed that the methods developed effectively prevented bacterial growth due to the existence of spatial barrier between the contaminated disc and the medium. On the contrary, the growth of hyphal strands of *Phytophthora* was not limited by the spatial barrier which grew further aerially, contacted the medium and established without any bacterial contamination.

Conclusions

The results emanated from the present study indicated that, zoospore encystment-based method employing baits like thermocol, impregnated paper discs, leaves of *Albizzia falcataria* and green gram roots that are cost effective and consumes lesser time for isolation could be used to isolate *Phytophthora* from infested rhizospheric soils of black pepper on modified carrot agar medium. Further, the two approaches of cabin sequestering methods which were found promising in obtaining pure cultures of *Phytophthora* devoid of bacterial contamination, can also be employed to retrieve bacterial contaminated cultures of research significance.

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Data availability The data that supports the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare no competing interests.

Ethical approval This article does not contain any studies with human participants or animals (vertebrates) performed by any of the authors.

Conflict of interest The authors declare that they have no conflict of interest in the publication.

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