Received: 2 February 2018 | Accepted: 19 April 2018

DOI: 10.1111/jph.12715

ORIGINAL ARTICLE

WILEY Journal of Phytopathology

Identification and characterization of *Neopestalotiopsis clavispora* associated with leaf blight of small cardamom (*Elettaria cardamomum* Maton)

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Funding information
Indian Council of Agricultural Research

Abstract

Leaf blight is a major foliar disease prevalent in all cardamom-cultivating tracts, manifesting in diverse forms of symptoms. In this study, six symptomatological variants were delineated based on the expression of foliar symptoms in cardamom genotypes (Malabar, Mysore and Vazhukka) and designated as SV 1 to SV 6. Among the symptomatological variants, SV 1, SV 2, SV 3 and SV 6 were more pronounced in Vazhukka, while SV 4 and SV 5 were prominent in Malabar type. Subsequent isolation from the variants yielded whitish colonies, which were correspondingly coded as SV 1 to SV 6. The conidia were fusiform, five-celled, with three median versicoloured cells, two terminal hyaline cells and measured 23.1–27.25 × 3.84–4.43 μm. The apical cells had two to three tubular, flexuous, unbranched appendages, whereas the basal appendage was single, tubular and unbranched. Based on conidial characteristics and molecular characterization with internal transcribed spacer rDNA region, partial β-tubulin, translation elongation factor 1 alpha and large subunit (28S) of the nrRNA genes revealed identity of the pathogens as *Neopestalotiopsis clavispora*. The pathogenicity test was performed on Malabar, Mysore and Vazhukka genotypes, and Koch's postulates were proved. *In-vitro* interaction at three temperature regimes indicated that *N. clavispora* was inhibitory to *Colletotrichum gloeosporioides* at 10 and 30°C. Among the fungicides, carbendazim, propiconazole and carbendazim-mancozeb completely arrested hyphal growth of *N. clavispora* under *in-vitro* conditions. This study constitutes first report on the association of *Neopestalotiopsis clavispora* with leaf blight disease of small cardamom.

KEYWORDS

cardamom, endophyte, fungicide sensitivity, leaf blight, *Neopestalotiopsis clavispora*, symptomatological variants

1 | INTRODUCTION

Small cardamom (*Elettaria cardamomum* Maton) is a commercially important Zingiberaceous spice originated and evolved in the biodiversity-rich evergreen forest ecosystem of Western Ghats, India. Besides its centre of origin, cardamom is widely cultivated in Guatemala, Sri Lanka, Papua New Guinea and Tanzania (Ravindran, 2002). Unprecedented interventions of multitude pernicious biotic and abiotic factors act as major impediments to realize appreciable yield levels and decline in gross productive area in cardamom. Several pathogenic microbes are reported to infect cardamom, inciting a variety of diseases with a potentiality to cause substantial economic loss. Among the foliar diseases of cardamom, leaf blight is distributed across all agroclimatic zones, inflicting damage

invariably to all the genotypes. Divergent species belonging to the hemibiotrophic ascomycetous pathogen, *Colletotrichum*, incites leaf blight (Chethana, Chowdappa, Biju, Praveena, & Sujatha, 2016), which generally exacerbates and assumes epiphytotic proportions during postmonsoon period (Thomas & Bhai, 2002). *Pestalotiopsis*, a conglomeration of highly assorted species-rich asexual genus, is distributed pan-tropical as well as temperate ecosystems (Sutton, 1980). *Pestalotiopsis* species are commonly encountered as endophytes and are reported to be rich resources of novel bioprospective molecules with agricultural, industrial and medicinal applications (Xu, Yang, & Lin, 2014). Several *Pestalotiopsis* species are reported to be opportunistic pathogens, invading plants especially under stressed conditions which is attributed to their ability to switch nutritional modes as pathogens (Hu, Jeewon, Zhou, Zhou, & Hyde, 2007; Maharachchikumbura et al., 2012) and saprobes on dead leaves, bark and twigs (Maharachchikumbura, Guo, Chukeatirote, Bahkali, & Hyde, 2011; Maharachchikumbura, Hyde, Groenawald, Xu, & Crous, 2014). *Pestalotiopsis* is reported to cause significant yield loss in a broad spectrum of economically important horticultural crops such as coconut, ginger, apple, guava, mango, blueberry, chestnut, grapevine, hazelnut, litchi, orchid, peach, rambutan and tea (Espinoza, Briceno, Keith, & Latorre, 2008; Valencia, Torres, & Latorre, 2011; Gonzalez et al., 2012; Maharachchikumbura et al., 2014; Chamorro, Aguado, & Santos, 2016). *Pestalotiopsis royenae* and *P. versicolor* incite foliar diseases in large cardamom (*Amomum subulatum*) which is also affiliated with Zingiberaceae (Srivastava & Verma, 1989a, 1989b). *P. clavispora* is also reported to infect the epithelial cells of human eye causing fungal keratisis (Monden et al., 2013). *Pestalotiopsis* is characterized by conidial morphology, septation, pigmentation of median cells, appendages originating from apical cell and basal appendage (Jeewon, Liew, & Hyde, 2002), which are considered as stable characters to taxonomically delineate the genus (Hu et al., 2007). Nevertheless, it is regarded as a complex genus which makes the identification challenging as inter/intraspecific variations in growth rate, conidial morphology and fruiting structures are common across different species. The taxonomy of *Pestalotiopsis* is ambiguous which warrants a critical analysis employing multigene phylogeny and 10 gene regions (ACT, β-tubulin, CAL, GPDH, GS, ITS, LSU, RPB1, SSU and TEF 1) were predominantly utilized to resolve cryptic *Pestalotiopsis* species among which, ITS, β-tubulin and TEF 1 proved to be the promising markers (Ismail, Cirvillere, & Polizzi, 2013; Maharachchikumbura et al., 2012). Our earlier inferences based on aetiological studies revealed the consistent association of an unidentified *Pestalotiopsis*-like genus with cardamom leaf blight besides the principal pathogen, *Colletotrichum*. Hence, to decipher the role of putative *Pestalotiopsis* associated with leaf blight, the present investigation was formulated with the objectives to characterize the diverse symptoms of leaf blight manifested on different genotypes, to identify and characterize putative *Pestalotiopsis* employing phenotypic traits and molecular approach, proving Koch's postulates, discern its endophytic nature and assess sensitivity towards broad spectra of fungicides.

2 | MATERIALS AND METHODS

2.1 | Categorizing symptomatological variants

To delineate the symptomatological variants and to develop a descriptor for each variant, foliar symptoms manifested on different genotypes of cardamom accessions maintained in the field gene bank, an *in-situ* germplasm conservatory of ICAR-Indian Institute of Spices Research Regional Station, Appangala, Madikeri, Karnataka, India (12°26′N Latitude, 75°45′E Longitude, 920 m above MSL), were recorded during October 2015 to September 2016. During the period of observation, temperature regime of the region ranged between 11° and 34°C with an annual rainfall of 2,800 mm which registered the peak during July to August.

2.2 | Collection, isolation and phenotypic characterization

The infected leaves belonging to each symptomatological variant from 120 cardamom accessions representing Malabar, Mysore and Vazhukka were collected. The infected leaves were subjected to initial surface sterilization by washing in sterilized water. The necrotic tissues along with peripheral healthy zones were incised from advanced margin of the lesions, dissected into bits (1 × 1 cm), surface sterilized with ethanol (70%) for 30 s followed by sodium hypochlorite (1%) for 2 min and washed with sterile distilled water consecutively three times (Lu et al., 2015). Later, the bits were aseptically transferred to potato dextrose agar (PDA) medium, incubated at 25°C with 12-hr photoperiod (alternating light and dark conditions) and observed periodically. The hyphal initials emerging out of the leaf bits were aseptically transferred to growth medium in Petri dishes and maintained at 25°C under continuous illumination for stimulating conidiation (Ibrahim, Satour, & Elakkad, 1976). Single-spore cultures were derived from each isolate culture, assigned the code SV (for Symptomatological Variant) and maintained at 4°C for subsequent studies. Observations on macrophenotypic features such as colony colour (top and reverse), margin, topography, zonation and growth rate were recorded by culturing the isolates on PDA medium. For recording phenotypic characters, mycelial plugs (5 mm) were aseptically punched out from the periphery of actively growing 7-day-old cultures of each isolate. The plugs were transferred to PDA in triplicates and incubated at temperature range of 25 ± 2°C. The mean radial mycelial growth (mm per day) of each isolate was recorded daily, and after 10 days, colony size, conidiation and colour were recorded. For examining conidial morphology, cultures were maintained at 25°C under continuous illumination for stimulating conidiation as described by Ibrahim et al. (1976). The conidia harvested from the culture plates of each isolate were mounted in water, stained with lactophenol cotton blue, and the size was measured at 10× magnification. The conidial dimensions were determined by measuring the length and width of 50 arbitrarily selected conidia of each isolate prepared in sterile distilled water under an Eclipse Ci

compound light microscope (Nikon Co. Ltd, Japan). The isolates were identified initially by comparing phenotypic and cultural characteristics based on size of conidia, colour, number, branching and length of apical appendages and length of basal appendage (Guba 1961).

2.3 | Molecular characterization

The genomic DNA of isolates was extracted from mycelial mat employing cetyl trimethyl ammonium bromide (CTAB) method (Knapp & Chandlee, 1996). The mycelial mat was harvested by filtration through sterile filter paper and stored at −70°C until DNA extraction. One gram of frozen mycelium was ground to fine powder in liquid nitrogen and incubated in 5 ml, 2% CTAB extraction buffer [10 mM trisbase (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, CTAB (2%)], mercaptoethanol (0.1%) and PVP (0.2%) at 65°C for 1 hr. The suspension was added with equal volume of phenol–chloroform–isoamyl alcohol (25:24:1) mixture, vortexed to mix two phases and centrifuged at 13,200 *g* for 5 min. The supernatant was transferred to a clean tube and mixed with equal volume of ice-cold isopropanol and incubated at 25°C for DNA precipitation. The precipitate was collected by centrifugation, and the pellet was washed with 0.1 M ammonium acetate in 70% ethanol and incubated for 15 min. The pellet was resuspended in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), and the DNA concentration was estimated using spectrophotometer (Genway Genova, Bibby Scientific Ltd., Dunmow, UK) at 620 nm.

2.4 | PCR amplification of multiple regions

For nucleotide sequence comparisons, the nuclear rDNA operon spanning the 3′ end of the 18S nrRNA gene, the first internal transcribed spacer region, the 5.8S nrRNA gene, the second internal transcribed spacer region and the 5′ end of the 28S nrRNA gene (ITS), the partial β-tubulin (TUB) and partial translation elongation factor 1-alpha (TEF 1) genes were amplified using primer pairs LR0R/LR5 (Rehner & Samuels, 1994; Vilgalys & Hester, 1990), ITS5/ITS4 (White, Bruns, Lee, & Taylor, 1990), T1/Bt-2b (Glass & Donaldson, 1995; O'Donnell & Cigelnik, 1997) and EF1-728F/EF-2 (Carbone & Kohn, 1999; O'Donnell, Kistler, Cigelnik, & Ploetz, 1998). The amplification conditions for LSU, ITS and TEF 1 were followed as per Crous et al. (2013) and for TUB, Lee, Groenewald, and Crous (2004). The analysis was carried out by adding 5 μl of loading buffer to 25 μl of PCR products, which were loaded onto 1.2% agarose gel (Himedia Laboratories Chemicals Ltd., India), electrophoresed at 5 V/cm and visualized under UV after staining the gel with ethidium bromide with 100 bp ladder (Merck India Ltd.) as the size marker. The PCR products were eluted using QIAquick Gel Extraction Kit—Qiagen according to the recommendations of the manufacturer, and DNA sequencing was performed at Sci Genome Ltd., Cochin, India. The sequence data were aligned, and similarity searches of the GenBank database were determined using the National Center for Biotechnology Information Blast Network Server. A multiple sequence alignment was constructed using CLUSTALX with gap opening penalty of 15 and gap extension penalty 6.66 (Hoffman & Baron, 1998). The phylogenetic analyses were carried out by neighbour-joining method using MEGA v. 5.2.2 (Saitou & Nei, 1987) for all the genes amplified. The sequence information of other species and related genera were retrieved from the NCBI nucleotide database for comparison and out grouping.

2.5 | Pathogenicity assay

To prove pathogenicity and Koch's postulates *in planta*, three varieties/accessions of cardamom, *viz*. Appangala 1, FGB 4 (Field Gene Bank 4) and Njallani Green Gold representing the three genotypes, *viz*. Malabar, Mysore and Vazhukka, respectively, were used. The cultures, SV 1 and SV 4 (the most common SVs) were used for inoculation. The clones of the test plants were established under controlled conditions, and the second fully opened succulent leaves were inoculated either with mycelial discs or conidial suspension employing pin-prick method. Before inoculation, the leaves were surface disinfested with sodium hypochlorite (0.5%) for 2 min, washed with sterile distilled water and blot dried. Further, 10 pricks were made with sterilized needles on either side of the midrib equidistantly. For inoculation with discs, mycelial plugs (5 mm) derived from the margins of 7-day-old culture were placed at the point of pin-prick injury (mycelial mat facing the leaf) on abaxial surface of the leaves over which moistened cotton was placed to provide adequate moisture and humidity. For inoculation with conidial suspension, the culture was exposed to 25°C under continuous illumination for enhancing conidiation. The conidia were harvested by adding 10 ml of sterilized distilled water onto the Petri dish and subjected to gentle swirling to dislodge conidia. The concentration of conidia was adjusted to 3×10^6 conidia/ml using haemocytometer and subsequently used as the standard density of inoculum for pathogenicity test. Further, 50 μl of conidial suspension was deposited on the wound, followed by placing a small bit of moistened cotton at the point of inoculation. The cotton bits were moistened by misting distilled water with hand-driven atomizer at an interval of 12 hr, till development of symptoms. The inoculated plants in both cases were maintained at 25 ± 2°C and 90 ± 5% relative humidity with 12 hr of photoperiod. The control plants received mycelial plugs devoid of fungal colony and sterile distilled water. The inoculated plants were regularly monitored for the manifestation of symptoms.

2.6 | Endophytic association and interaction with *Colletotrichum gloeosporioides*

To analyse the role of putative *Pestalotiopsis* as endophyte, the experiment was undertaken during October 2015 to September 2016. Six gene bank accessions (representing three genotypes) expressing distinct SV 1 to SV 6 symptoms were selected and tagged (each accession had five clumps with 10 to 15 aerial tillers). Apparently healthy plant samples comprising leaves and portion of aerial tillers

were collected at monthly intervals for 12 months, and isolation was carried out following the procedure outlined by Liu, Wu, and Xu, (2007) with modifications. The apparently healthy leaves and bits of aerial tillers were washed under running tap water, sterilized with 75% ethanol (60 s) followed by 1% sodium hypochlorite (5 min) and 75% ethanol (30 s). Samples were washed thrice with sterilized water, dissected into pieces (1 × 1 cm), placed on PDA medium and incubated at 25°C. To investigate the interaction between different isolates and *Colletotrichum gloeosporioides* (Appangala isolate), respective cultures were placed on PDA medium confronting each other and maintained at 10, 20 and 30°C. The temperature regimes were considered based on the average maximum (T_{max}) and minimum (T_{min}) temperatures usually prevailing under field conditions. To avoid lag effect, the plates were maintained at respective temperature 24 hr before inoculation with the cultures. Observations on radial growth were recorded up to 10 days postinoculation, and per cent inhibition was calculated.

2.7 | Fungicide sensitivity

Sensitivity of the isolates was tested against eight fungicides, which was selected based on criteria such as recommended fungicides, toxicity class (green and blue) and other molecules. Among the chemicals evaluated, Bordeaux mixture, carbendazim, mancozeb and carbendazim-mancozeb were commonly recommended and green labelled (except Bordeaux mixture), whereas propineb, dimethomorph, propiconazole and fenamidone-mancozeb were blue labelled and not recommended to manage cardamom leaf blight. The commercial formulations of the test fungicides were evaluated under *in-vitro* conditions employing poisoned food technique. The fungicides were tested at six different concentrations, *viz*. 0.05%, 0.125%, 0.25%, 0.5%, 1% and recommended dose. Fungicidal suspensions were prepared by dissolving requisite quantities of each fungicide in warm PDA, and 20 ml of the medium was poured into Petri dishes and medium without fungicide served as control. Mycelial discs (5 mm diameter) derived from the advanced margin of 7-day-old culture of the isolates were placed at centre of each Petri dish, and each treatment was replicated thrice. The plates were incubated at 25 ± 2°C, and observations on radial growth of the colony were recorded 7 days after the incubation period. The per cent inhibition of the colony growth was calculated using the formula:

$$\frac{C-T}{C}\times 100$$

where C = growth of culture in control plate, T = growth of culture in fungicide-treated plate.

2.8 | Data analysis

The *in-vitro* bioassay experiments were laid out in completely randomized design (CRD), the per cent data were transformed using arcsine transformation, and statistical analysis was carried out using the software package AGRES version 7.01 @ 1994 Pascal Intl Software Solutions.

3 | RESULTS

3.1 | Categorization of symptomatological variants

The foliar symptoms developed on 120 field gene bank accessions representing all genotypes were recorded and based on symptom expression six variants were delineated and designated with code numbers SV 1 to SV 6. The symptoms manifested on adaxial and abaxial surfaces on the foliage are described (Table 1, Figure 1). Among the symptomatological variants, SV 1, SV 2, SV 3 and SV 6 were more pronounced in Vazhukka, while SV 4 and SV 5 were prominent in Malabar. The predominant symptom (SV 4) included formation of brownish streaks with yellow halo which later turned brownish with extensive coalition advancing towards proximal and distal ends leading to foliar blight. The severity of foliar infections was comparatively low during the monsoon (June to September) which attained the peak during postmonsoon period.

3.2 | Phenotypic characterization

The colony characteristics of putative pathogen associated with different symptomatological variants varied significantly. The isolates representing SV 2, SV 3 and SV 5 were whitish, and SV 1, SV 4 and SV 6 were whitish to pale honey. The substrate colour of SV 3, SV 4, SV 5 and SV 6 was yellowish, whereas SV 2 and SV 1 were whitish and brownish, respectively. SV 1 and SV 3 had crenate margins, while others had smooth. Topography of the colonies was predominantly raised and fluffy, and zonations were observed in all the isolates. Maximum growth was observed in isolate SV 2 (90 mm), and the minimum was observed in SV 5 (75 mm) on 7th day postinoculation (Table 2, Figure 2a, b). The conidia were fusiform, five-celled (quinquiloculatae), versicoloured with three olivaceous brown median cells, two apical and basal hyaline cells (Figure 2c). The apical cells had two to three flexuous, unbranched appendages, and basal appendage was solitary, tubular and unbranched. The average conidial length ranged from 23.1 to 27.25 μm with maximum in SV 5, and conidial width ranged between 3.84 and 4.43 μm (average), with maximum in SV 1. The length of apical appendages varied from 12.0 to 23.55 μm (average), and maximum was observed in SV 6, whereas the average length of basal appendages was in the range of 2.8 to 4.65 μm with maximum length recorded in isolate SV 3 (Table 3). The spores extruded as blackish gelatinous mass randomly distributed as exudations on the colony surface (Figure 3a). The pycnidial conidiomata were globose, solitary or aggregated, embedded or semi-immersed on growth medium, and the conidiophores were indistinct and reduced to

TABLE 1 Characterization of symptomatological variants

Symptomatological variant code	Symptomatology	
	Adaxial surface	Abaxial surface
SV 1	Symptoms initiated as minute yellow spots leading to dark yellow isolated spots. In the advanced stage, minute whitish brown spot developed at centre of the spot	Pale yellow spots
SV 2	Symptoms initiated as narrow elongated streaks with brown margin which elongated towards leaf margin leading to shredding of the leaves. In later stages, large blighted areas developed along the shredded areas	Narrow elongated streaks leading to shredding of leaves
SV 3	Symptoms developed as brown spots with dark brown margin surrounded by yellow hallow. In later stages, grey centre with minute black dots developed leading to shredding of grey centre and formation of shot holes	Dark brown spots with grey centre and shot hole symptom
SV 4	Symptoms initiated as rectangular brown streaks surrounded by yellow margin. In the later stages, the streaks turn brownish white with extensive elongation on both the ends. Large blight symptoms are developed due to coalescing of streaks	Pale brown streaks surrounded by yellow margin
SV 5	Symptoms manifested as white rectangular streak with dark brown margin. In the later stage, papery white patches with minute black dots developed leading to shredding along middle portion of the streak. White streaks coalesced to form blighted areas	Pale creamish white spots with brown margin
SV 6	Symptoms developed as dark brown spots surrounded by yellow hallow. In the later stages, shot hole symptoms are observed	Pale brown spots with shot hole symptoms

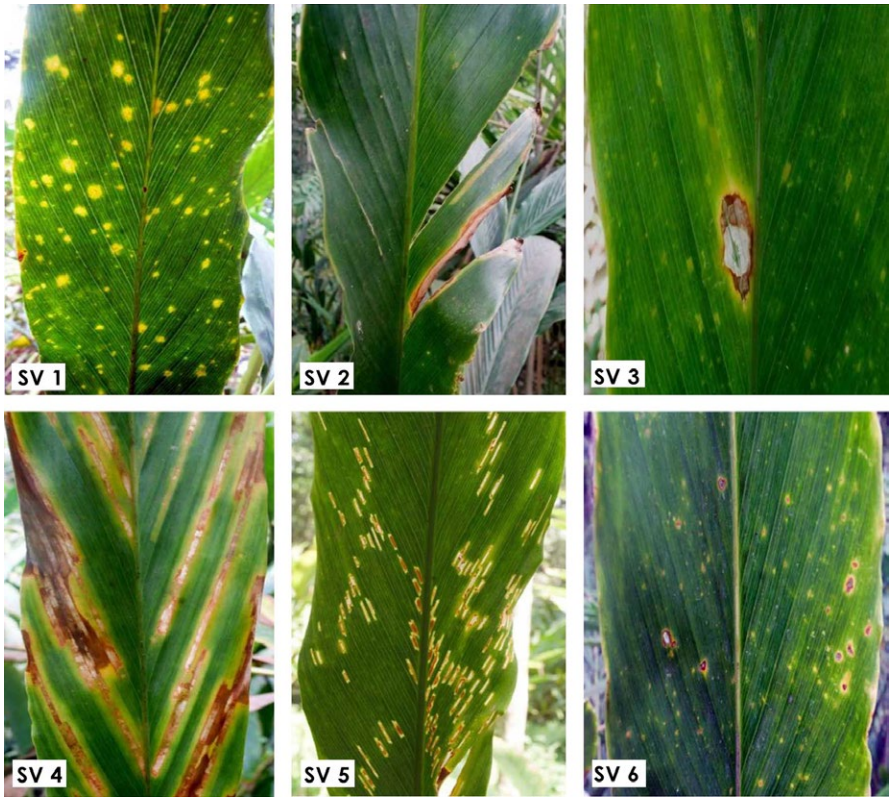

FIGURE 1 Symptomatological variation in small cardamom leaf blight [Colour figure can be viewed at wileyonlinelibrary.com]

conidiogenous cells (Figure 3b). The spore germination initiated from the basal versicolourous cell which swelled (Figure 4a), became spherical leading to the emergence of hyaline germ tube (Figure 4b).

3.3 | Molecular characterization

Molecular characterization was carried out employing the internal transcribed spacer (ITS) rDNA region, partial β-tubulin (TUB),

TABLE 2 Phenotypic characteristics of *Neopestalotiopsis clavispora*

Isolate Code	Colony		Margin	Topography	Zonation	Colony diameter (mm)
	Top	Reverse				
SV 1	White to pale yellow	Brown	Crenate	Raised and fluffy	Present	75
SV 2	White	White	Smooth	Raised and powdery	Present	90
SV 3	White	Light yellow	Crenate	Raised and fluffy	Present	80
SV 4	White to pale honey	Yellow	Smooth	Raised and cottony	Present	80
SV 5	White	Yellow	Crenate	Raised and fluffy	Present	70
SV 6	White to pale honey	Yellow	Crenate	Raised and fluffy	Present	75

translation elongation factor 1 alpha (TEF) and large subunit (28S) of the nrRNA genes amplified, T1/Bt-2b, EF1-728F/EF-2 and LR0R/LR5, respectively. The PCR product was approximately 550 bp for all isolates with the primers ITS5/ITS4 (Figure 5a), for the primer LR0R/LR5 at 950 bp (Figure 5b), for the primer T1/Bt-2b at 850 bp (Figure 5c) and for the primer EF1-728F/EF-2 at 500 bp (Figure 5d). High-quality sequences of two isolates, *viz*. SV 1 and SV 4 (selected based on the most common symptoms) were used for generating phylogeny tree and comparison using MEGA v. The PCR product sequence was compared by BLAST search (Altschul, Gish, Miller, Myers, & Lipman, 1990), the isolates were confirmed to the species level, and LSU of rRNA partial gene sequences (KY 427127.1 and KY 427328.1) and ITS (MG 386208 and MG 386209) were deposited with NCBI. A phylogenetic tree was generated from the analysis of the aligned sequences of the ITS region with the primers ITS5/ITS4 in this study. The tree separated *Pestalotiopsis* from other reference species in distinct clusters, with one clade consisting of all the *Pestalotiopsis* species. *Pestalotiopsis clavispora* was found in close proximity of almost 100 per cent similarity with isolate SV 1 and SV 2 (Figure 6). Likewise, phylogenetic tree generated with the nrRNA (LSU) gene also showed maximum similarity with *P. clavispora* which further confirmed identity of the pathogen (Figure 7).

3.4 | Pathogenicity assay

The experiment designed to prove Koch's postulates showed that inoculation with the spore suspension favoured the infection process compared with mycelial plug method. The symptoms were initiated 15 days postinoculation and attained prominence approximately 30 days after inoculation characterized with lesions with brownish centre surrounded by yellow halo in Malabar (Figure 8a), Mysore (Figure 8b) and Vazhukka (Figure 8c) genotypes. Later, Koch's postulates were proved by reisolating the pathogen from symptomatic lesions.

3.5 | Endophytic association and interaction with *Colletotrichum gloeosporioides*

The analyses for endophytic association indicated that SV 1, SV 2, SV 3 and SV 6 were consistently associated as endophytes during the period of investigation and SV 4 could be isolated during October 2015 to May 2016, whereas SV 5, the rarest symptomatological variant, could be isolated only during December 2015 to February 2016. In general, all the SVs were isolated during December 2015 to February 2016, irrespective of the genotypes with higher colonization rates in leaves (data not presented). The interaction studies at different temperature regimes indicated that, at 10°C, *N. clavispora* and *C. gloeosporioides* attained 44.66 mm and 32.66 mm, respectively, and SV 5 inhibited *C. gloeosporioides* up to 51.99% with an apparent demarcating inhibition zone (Figure 9a). At 20°C, both the pathogens attained 70 mm with 70% inhibition by SV 5 which arrested advancement of growth of *C. gloeosporioides*. While at 30°C, *C. gloeosporioides* attained 70 mm and *N. clavispora* 58 mm with an evident 63.81% inhibition by SV 2 due to arresting hyphal growth and formation of inhibition zone (Figure 9b).

3.6 | Fungicide sensitivity

Among the molecules evaluated, carbendazim, propiconazole and carbendazim-mancozeb completely inhibited hyphal growth of *N. clavispora* at all the concentrations assessed. Bordeaux mixture, the commonly recommended fungicide against foliar diseases of cardamom, was comparatively less effective at recommended dose. Among the other molecules, fenamidone-mancozeb and mancozeb were found promising (Figure 10).

4 | DISCUSSION

Small cardamom, the commercially important export-oriented spice, is highly valued for its intrinsic superior qualities such as flavour and aroma. Several soil/air/water/vector-borne diseases incited by oomycetes, fungi, viruses, bacteria and nematodes inflicting damage to aerial as well as subterranean plant parts are considered as the major production constraints in cardamom in India and elsewhere. Among the foliar diseases, leaf blight (*Chenthal*), earlier considered as a minor disease, is reported to be emerging as a major threat to cardamom cultivation (Thomas & Bhai, 2002). The causal agent of

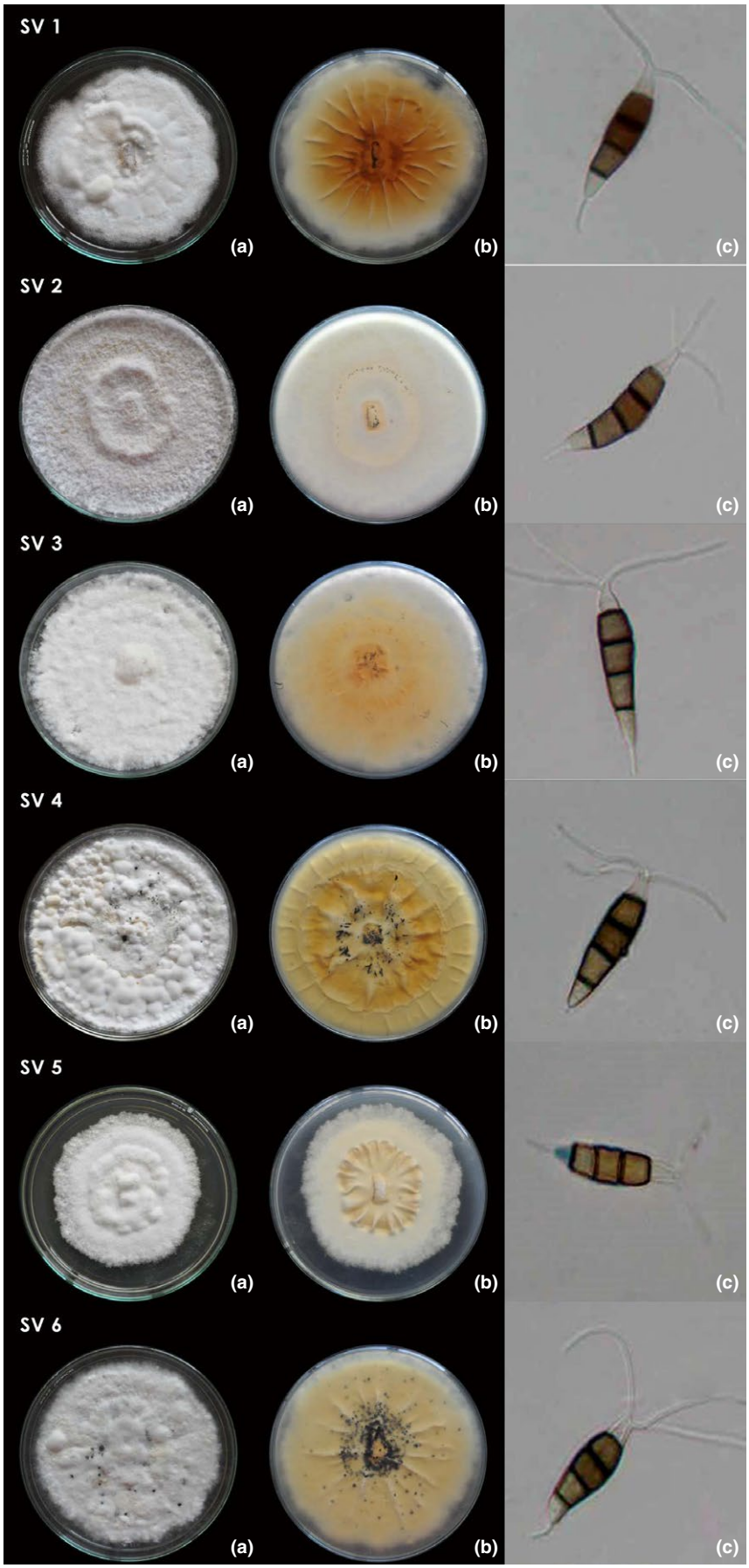

FIGURE 2 Phenotypic features of *Neopestalotiopsis clavispora*. (a) colony on PDA—top, (2) colony on PDA—reverse and (c) Conidia [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 3 Conidial characteristics of *Neopestalotiopsis clavispora*

Isolate Code	Conidial characteristics
SV 1	Five-celled, fusiform, basal cell hyaline (3.9–4.8 × 2.4–2.9 μm), versicolourous, second cell from base (4.7–4.9 × 4.4–5.1 μm), third cell (4.3–5.3 × 5.8–5.9 μm), fourth cell (4.6–5.1 × 5.1–5.5 μm), apical cell subcylindrical, hyaline (3.8–4.8 × 2.6–3.8 μm), two to three tubular unbranched apical appendages (10.9–33.7 μm), basal appendage single, unbranched (3.4–5.2 μm)
SV 2	Five-celled, fusiform, basal cell hyaline (3.0–4.9 × 2.4–2.9 μm), versicolourous, second cell from base (4.0–6.2 × 4.2–5.0 μm), third cell (4.8–6.2 × 5.2–5.8 μm), fourth cell (5.0–5.7 × 5.0–5.5 μm), apical cell subcylindrical, hyaline (3.3–5.1 × 1.6–2.0 μm), three tubular apical unbranched appendages (16.1–7.9 μm), basal appendage single, unbranched (2.6–3.0 μm)
SV 3	Five-celled, fusiform, basal cell hyaline (3.6–6.4 × 1.8–3.0 μm), versicolourous, second cell from base (5.8–6.6 × 4.4–5.0 μm), third cell (5.1–5.4 × 5.2–5.9 μm), fourth cell (5.2–5.9 × 5.2–5.5 μm), apical cell subcylindrical, hyaline (3.5–4.2 × 2.6–3.1 μm), three tubular unbranched apical appendages (19.6–21.9 μm), basal appendage single, unbranched (4.1–5.2 μm)
SV 4	Five-celled, fusiform, basal cell hyaline (3.0–6.6 × 1.8–3.9 μm), versicolourous, second cell from base (5.2–6.5 × 3.4–4.7 μm), third cell (4.2–6.7 × 4.1–6.1 μm), fourth cell (4.5–5.9 × 3.9–5.6 μm), apical cell subcylindrical, hyaline (2.6–4.4 × 1.9–3.0 μm), three tubular unbranched apical appendages (14.0–23.5 μm), basal appendage single, unbranched (2.6–5.4 μm)
SV 5	Five-celled, fusiform, basal cell hyaline (4.8–5.6 × 1.8–1.9 μm), versicolourous, second cell from base (5.3–5.6 × 3.7–3.8 μm), third cell (4.7–6.0 × 5.5–6.0 μm), fourth cell (5.9–6.3 × 4.3–5.8 μm), apical cell subcylindrical, hyaline (4.2–6.1 × 2.8–3.4 μm), two to three tubular unbranched apical appendages (11.8–18.1 μm), basal appendage single, unbranched (3.0–8.0 μm)
SV 6	Five-celled, fusiform, basal cell hyaline (3.8–5.6 × 2.4–2.8 μm), versicolourous, second cell from base (4.2–5.6 × 3.9–4.4 μm), third cell (4.1–5.7 × 5.3–5.8 μm), fourth cell (4.6–5.6 × 4.3–5.1 μm), apical cell subcylindrical, hyaline (3.8–4.4 × 2.1–4.4 μm), three tubular unbranched apical appendages (15.9–31.2 μm), basal appendage single, unbranched (3.9–4.7 μm)

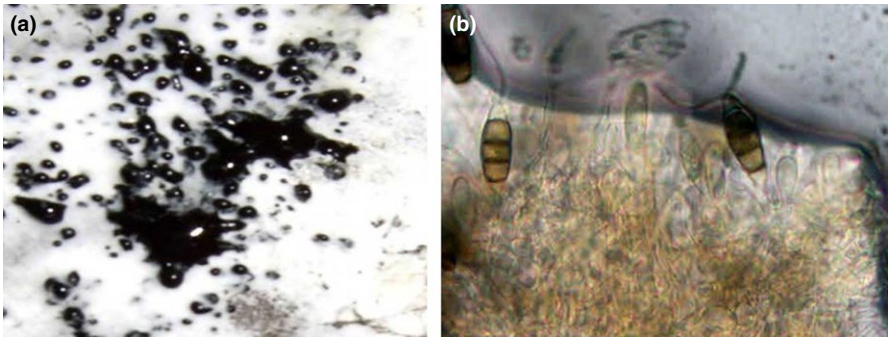

FIGURE 3 (a) Blackish conidial exudation of *Neopestalotiopsis clavispora* and (b) embedded conidiomata of *Neopestalotiopsis clavispora* on PDA [Colour figure can be viewed at wileyonlinelibrary.com]

leaf blight was earlier reported as *Corynebacterium* sp. (George & Jayashankar, 1977). However, detailed investigations on symptomatology, aetiology and management evidently proved that the disease is caused by *Colletotrichum gloeosporioides* (Govindaraju, Thomas, & Sudharsan, 1996). In recent times, Chethana et al. (2016) reported the association of divergent species of *Colletotrichum*, *viz*. *C. karstii*, *C. gloeosporioides*, *C. siamense*, *C. syzygicola* and *C. guajavae* with leaf blight of cardamom as elucidated through morphometric and multilocus phylogenetic analyses. Among the spices, association of *Pestalotiopsis* with larger cardamom (Srivastava & Verma, 1989a,b) and ginger (Maharachchikumbura et al., 2014), both the members of Zingiberaceae have been reported. Nevertheless, the current understanding on the association of *Neopestalotiopsis* with leaf blight of cardamom is ambiguous, as *Colletotrichum* species have already been

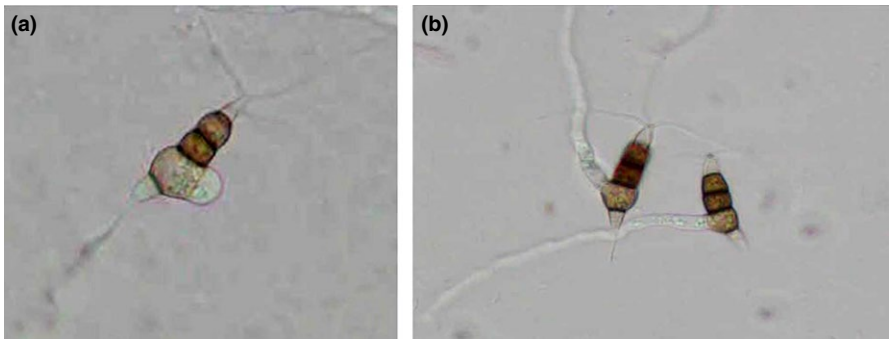

FIGURE 4 Germination of *Neopestalotiopsis clavispora*. (a) swelled basal versicolourous cell and (b) formation of germ tube [Colour figure can be viewed at wileyonlinelibrary.com]

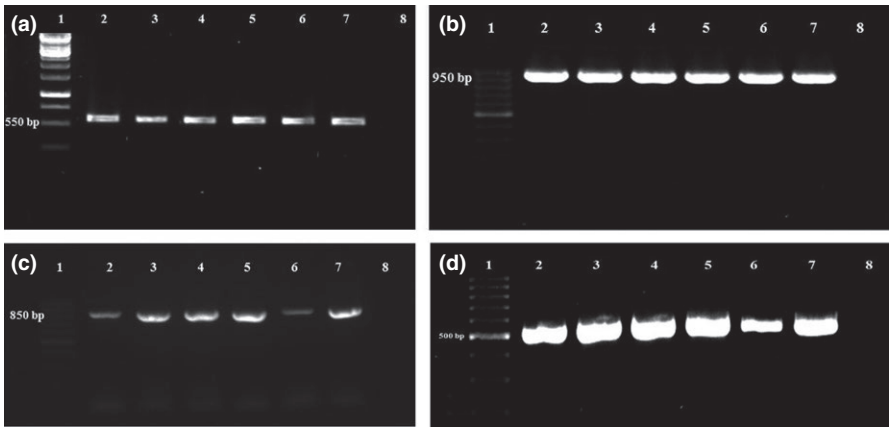

FIGURE 5 Amplification profiles of *Neopestalotiopsis clavispora* with different gene regions. (a) ITS, (b) LSU, (c) TUB and (d) TEF

reported as causative agents of the disease. *Pestalotiopsis* spp. is a common pathogen in areca nut (*Areca catechu*) and coconut (*Cocus nucifera*) that are cultivated in close proximity to cardamom fields, with different species involved. Keith, Velasquez, and Zee, (2006) reported that *Pestalotiopsis*-incited diseases in commercial guava orchards were aggravated due to the cultivation of wild guava and tea in contiguous with guava orchards. Identification of Pestalotioid fungi employing phenotypic markers is challenging (Hyde, McKenzie, & KoKo, 2011). Although taxonomy of Pestalotioid pathogens is confusing, significant advances have been achieved in the analysis of population structure, encompassing development and application of novel nucleic acid-based tools and integrating genomic computational approaches to unambiguously decipher the associated species, including several cryptic assemblages. Steyaert (1949) (in Maharachchikumbura et al. 2014) introduced the genus *Pestalotiopsis* to accommodate five-celled conidial forms of *Pestalotia*. *Pestalotiopsis* is reported to be highly variable in nature, and most of the species are morphologically indistinguishable. Several phenotypic traits of the genus *Pestalotiopsis* are extremely plastic and depend mostly on cultural and environmental conditions, which are rarely standardized (Maharachchikumbura et al., 2014). The genus *Pestalotiopsis* is taxonomically characterized primarily based on conidial morphology, and majority of the species are divided into different groups based on the size of conidia (Maharachchikumbura et al., 2011) The conidial morphometrics (length and width) are considered as excellent taxonomic markers to classify *Pestalotiopsis* (Hu et al., 2007). In our study, considerable variations were observed in conidial dimensions; however, generally the conidia were fusiform, five-celled, four septate, with three median versicoloured cells and two terminal hyaline cells. Palou, Montesinos-Herrero, Guardado, and Taberner (2013) described conidial characters of *P. clavispora* the causal agent of postharvest fruit rot of loquat with fusiform conidia (26 × 8 μm), five-celled with hyaline apical and basal cells, presence of dark brown median cells with one short basal and two to four long apical appendages, and existence of variation in conidial size was also reported (Hu et al., 2007; Wei, Xu, Guo, Liu, & Pan, 2005; Wei et al., 2007). In the present study, phenotypic characteristics of the isolates exhibited considerable variations which may be due to their genetic plasticity.

The pathogenicity on three genotypes indicated that *N. clavispora* could be a weak pathogen on cardamom as it presumably required an artificial portal of entry (through pin-prick injury) to initiate the process of pathogenesis and approximately 30 days for complete expression of characteristic symptoms. It is assumed that endophytic nature and horizontal transmission through planting materials of the pathogen might have adversely affected its capability to infect the host under natural conditions. Creating the points of entry artificially through pin-prick wounds allows the pathogenic isolate for internal access to the leaves and enhances infection. This study is consistent with inoculation studies by Baayen and Schrama (1990) in carnation, Buckley, Williams, and Windham (2009) in corn and Priyanka, Gohar, and Kumar (2013) in Indian mustard.

Molecular systematics has been successfully used in studies of this genus and has resulted in well-defined delineations of species (Crous et al., 2013; Lee et al., 2004). In the present study, molecular

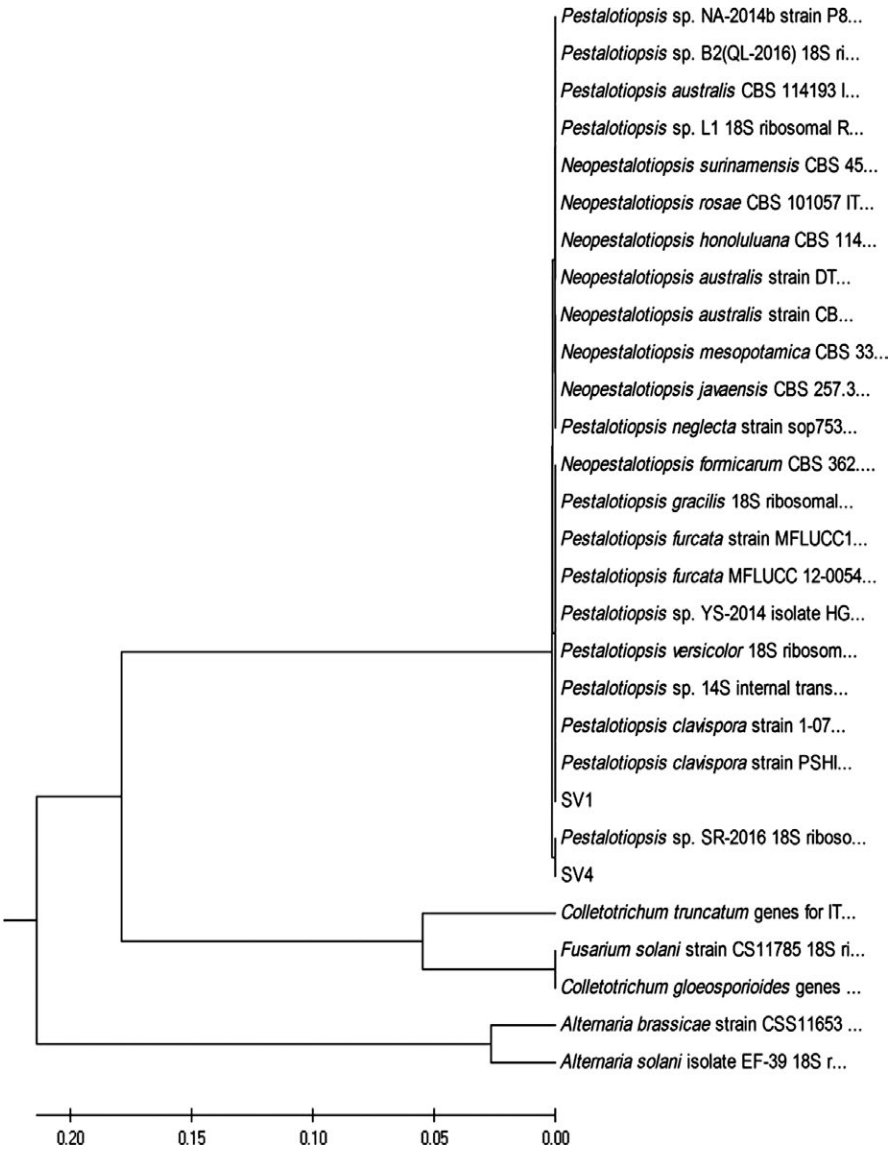

FIGURE 6 UPGMA analysis of internal transcribed spacer of *Neopestalotiopsis clavispora* with related species from GenBank

characterization was carried out employing the internal transcribed spacer (ITS) rDNA region, partial β-tubulin (TUB), translation elongation factor 1 alpha (TEF) and large subunit (28S) of the nrRNA genes amplified, T1/Bt-2b, EF1-728F/EF-2 and LR0R/LR5, respectively. The PCR product was approximately 550 bp for all isolates with the primers ITS5/ITS4, for the primer T1/Bt-2b amplified at 850 bp, for the primer EF1-728F/EF-2 at 500 bp and for the primer LR0R/LR5 at 900 which are in concordance with earlier reports (Crous et al., 2013; Lee et al., 2004). The ITS regions of nuclear rDNA have been reliable targets for the identification, differentiation and phylogenetic analysis of fungi using molecular techniques (Dunne, Glen, Tommerup, Shearer, & Hardy, 2002). The phylogenetic tree developed using the isolates (SV 1 and SV 4) clearly divided into two clusters, with one clade consisting all the *Pestalotiopsis* spp. *P. clavispora*. was found in close proximity of almost 100 per cent similarity with isolate SV 1 and SV 2 for both ITS and LSU regions. Based on molecular data using ACT, β-tubulin, CAL, GPDH, GS, ITS, LSU, RPB 1, SSU and TEF 1 genes, 14 new species (*Pestalotiopsis asiatica*, *P. chinensis*, *P. chrysea*, *P. clavata*, *P. diversiseta*, *P. ellipsospora*, *P. inflexa*, *P. intermedia*, *P. linearis*, *P. rosea*, *P. saprophyta*, *P. umberspora*, *P. unicolor* and *P. verruculosa*) and three epitypified species (*P. adusta*, *P. clavispora* and *P. foedans*) were described by Maharachchikumbura et al. (2012). The conidial as well as molecular-based evidences clearly indicate that the pathogenic genus associated in addition to *C. gloeosporioides* with leaf blight disease of cardamom is *Neopestalotiopsis clavispora*. *Neopestalotiopsis*, evolutionarily related to *Pseudopestalotiopsis* lineage characterized with concolourous median cells, has been carved out from *Pestalotia* to accommodate the genus with versicoloured median cells (Maharachchikumbura et al., 2014). The species described under *Pestalotiopsis* are identified based on five-celled, fusiform conidia, with three concolourous median cells and with hyaline end cells, while *Neopestalotiopsis* genus is distinguished from *Pestalotiopsis* based on versicolourous median cells (Maharachchikumbura, Larignon, Hyde, Al-Sadi, & Liu, 2016).

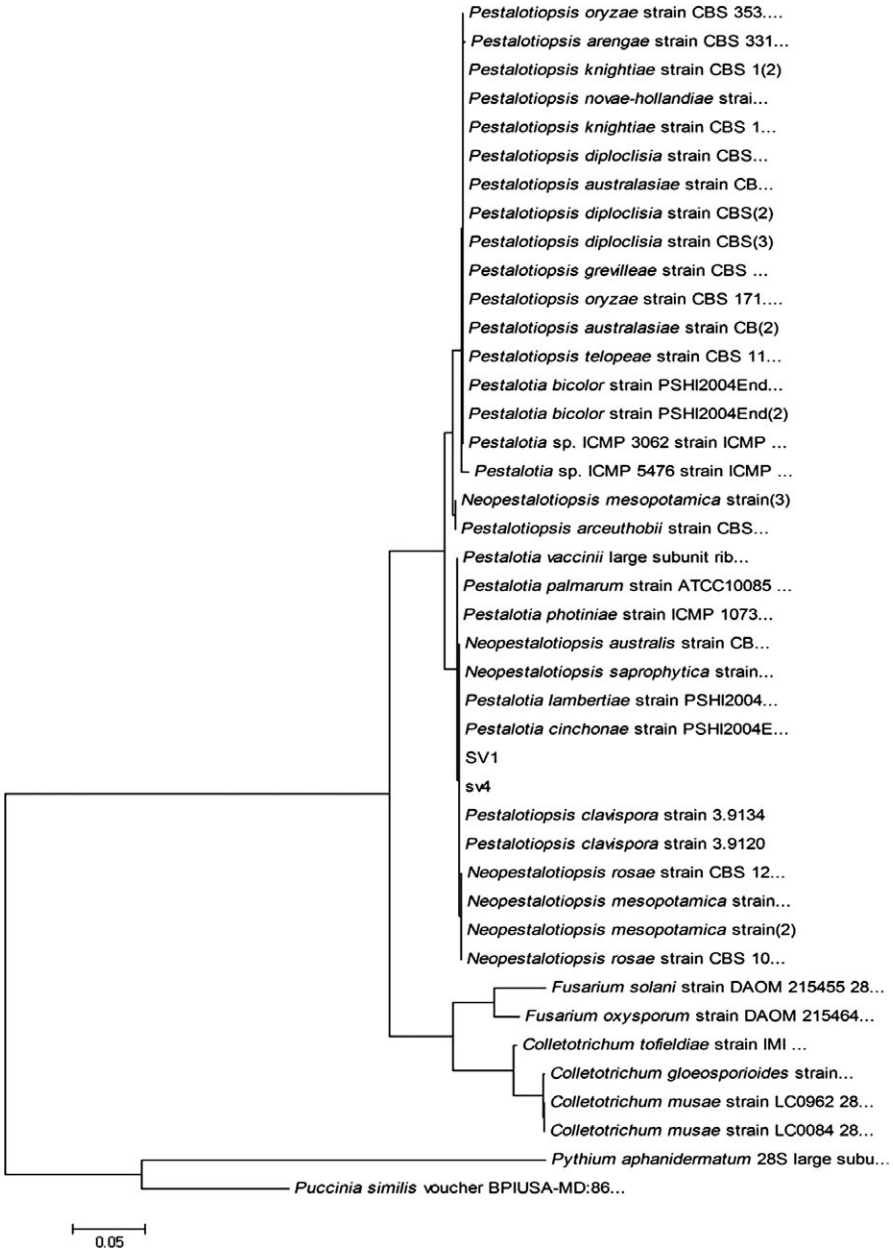

FIGURE 7 UPGMA analysis of large subunit (28S) of the nrRNA gene of *Neopestalotiopsis clavispora* with related species from GenBank

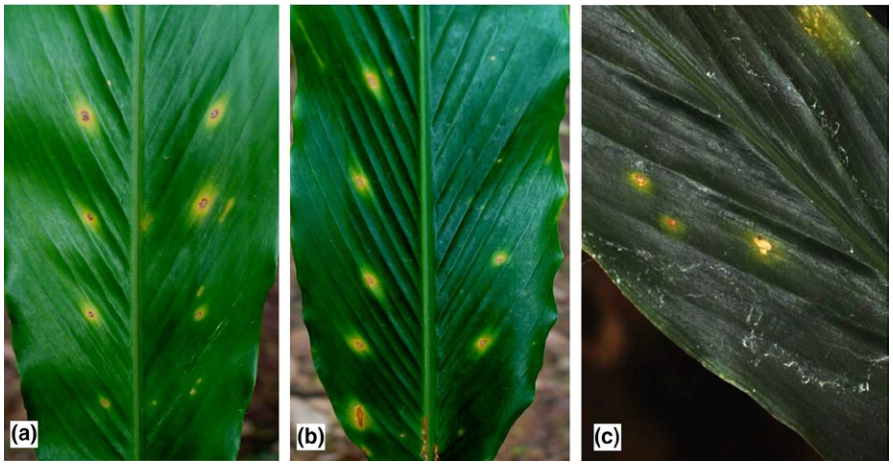

FIGURE 8 Pathogenicity assay: manifestation of symptoms on (a) Malabar, (b) Mysore and (c) Vazhukka [Colour figure can be viewed at wileyonlinelibrary.com]

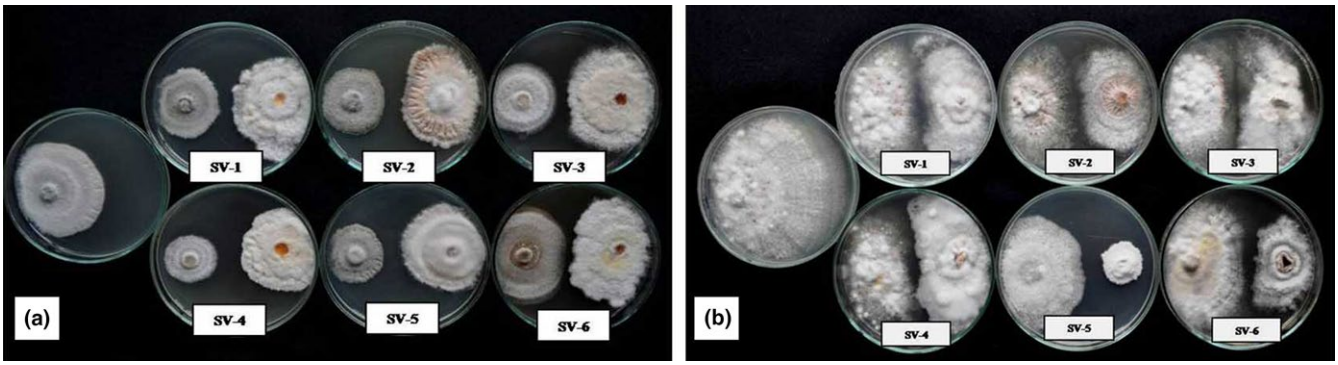

FIGURE 9 *In-vitro* interaction of *Neopestalotiopsis clavispora* with *Colletotrichum gloeosporioides* (a) at 10°C and (b) at 30°C [Colour figure can be viewed at wileyonlinelibrary.com]

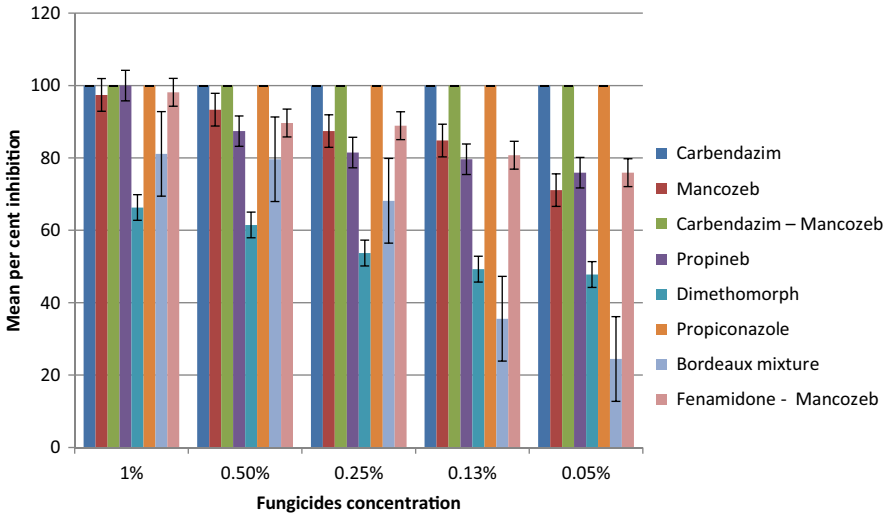

FIGURE 10 Sensitivity of *Neopestalotiopsis clavispora* to fungicides under *in-vitro* conditions [Colour figure can be viewed at wileyonlinelibrary.com]

In geoclimatic perspective, assemblages of *Pestalotiopsis* species are widely distributed in tropical regions as resident colonizers of internal architectural niches in plant communities of generally unrelated taxonomic lineages in a non-host-specific mode besides inhabiting a broad array of substrates as pathogens as well as saprophytes. Adaptability on diverse living or inert substrates unequivocally attests its superior ecological amplitude probably attributed to counteract antimicrobial bioactive molecules secreted by the host plants and capability of switching nutritional modes aided by myriad of secondary metabolites (Reddy, Murali, Suryanarayanan, Rajulu, & Thirunavukkarasu, 2016). Wei et al. (2007) reported *Pestalotiopsis clavispora* as endophyte in Theaceae and Podocarpaceae and further identified *P. karstenii*, *P. lawsoniae* and *P. theae* as endophytes capable of transforming into pathogenic lifestyle. Brown, Hyde, and Guest (1998) reported the pathogenic species; *C. gloeosporioides*, *C. musae* and *Phyllosticta musicola* as components of endophytic fungal communities of *Musa acuminata*. *Deightoniella torulosa*, the leaf spot pathogen, was also reported as an endophyte in wild banana (Photita, Lumyong, Lumyong, McKenzie, & Hyde, 2004). Considering the consistent association (with exception in few instances) of *N. clavispora* during different months, it is hypothesized that it is a cryptic endophyte with pathogenic potentialities colonizing the internal niche of small cardamom. The pathogenic transformation from quiescent endophytic to pathogenic phase might be triggered due to (a) alteration in the host/endophyte physiology as mediated through external stimuli such as erratic shift in temperature pattern consequently inducing stress in host or (b) secondary colonization of the moribund tissues previously invaded by *Colletotrichum*. The balanced symbiotic host–microbe coexistence is influenced by variations in weather variables, genetic make-up of host, the microbial taxa associated and stages of interaction which might lead to an irreversible shift in biology and adoption of contrasting lifestyles by the endophytes; completing life cycle as pathogens on some hosts, while surviving as mutualists or commensals on others (Stergiopoulos & Gordon, 2014). Among the weather variables, temperature plays a pivotal role in pathogenesis. The severity of leaf blight of cardamom is aggravated by increase in temperature (during postmonsoon period) and under open (less shaded) conditions (Thomas & Bhai, 2002). The information generated from the interaction studies indicates that, at low temperature, *N. clavispora* inhibited *C. gloeosporioides* apparently with the action of putative secondary metabolites. While, at high temperature, *C. gloeosporioides* was inhibited in confrontation with *N. clavispora*, the growth rate of former was higher compared to latter. Upadhyay and Dwivedi (1980) reported that the growth of *Pestalotiopsis funerea*, the leaf spot pathogen of *Eucalyptus globulus*, was optimum at 25°C. Likewise, temperature range of 25–32°C was

reported to enhance growth and increase severity of a variety of diseases incited by *C. gloeosporioides* in yam (Abang, Winter, Mignouna, Green, & Asiedu, 2003), mango (Dodd, Estrada, Matcham, Jeffries, & Jeger, 1991), sugar cane (Imtiaj, Alam, Islam, Alam, & Lee, 2007) and white beans (Tu, 1981). Xu et al. (2014) indicated several species within *Pestalotiopsis* secretes biologically active, structurally complex secondary metabolites possessing antifungal and antimicrobial properties. Hanada et al. (2010) reported antagonistic activity of the endophytic colonizer, *Pestalotiopsis* in *Theobroma cacao* and *T. grandiflorum* against *Phytophthora palmivora*, the black pod pathogen of cocoa.

A broad spectrum of fungicides endowed with single- and multiple-site inhibitory activities have been reported to manage diseases incited by phytopathogenic species of *Neopestalotiopsis* infecting several commercial horticultural crops. Based on inhibitory activity under *in-vitro* as well as field conditions, triazoles, benzimidazoles, carbamates, strobilurins, quinone inhibitors and copper-based fungicides have been reported to be effective against various *Pestalotiopsis* species (Palomar & Betonio, 1982; Sanjay, Ponmurugan, & Baby, 2008; Saju, Mech, Deka, & Biswas, 2011; Yamada and Sonoda 2012; Zhang et al., 2012; Rahman, Adhikary, Sultana, Yesmin, & Jahan, 2013; Yong, Chen, Fang, & Chung, 2014) in a variety of crop species. Saju et al. (2011) reported superiority of carbendazim over copper oxychloride, carbendazim-mancozeb and mancozeb under *in-vitro* conditions against *Pestalotiopsis* sp. causing leaf streak disease of large cardamom. Zhang et al. (2012) deduced the baseline sensitivity of *P. microspora*, the nut black spot pathogen of Chinese hickory to pyraclostrobin, a quinone-inhibiting fungicide. Yamada and Sonoda (2012) while analysing the resistance pattern in *P. longiseta* opined that recurring application of quinone inhibitor fungicides might lead to evolution of resistant strains owing to their single-site mode of action due to single amino acid substitutions in the QoI-targeted cytochrome b protein. Yong et al. (2014) reported low fungicide-sensitive isolates of *Pestalotiopsis*, the guava scab pathogen with point mutation in codon 198 (GAG → GCG) of β-tubulin gene leading to evolution of benzimidazole-resistant phenotypes. Benzimidazole, carbamates and copper-based fungicides have been recommended to manage leaf blight disease in cardamom (Thomas & Bhai, 2002). The results emanated from the present study are also in conformation with earlier reports. However, deriving a schedule of fungicide application amalgamating molecules belonging to benzimidazole/propiconazole group (systemic and single point mode of action) alternating with copper-based/dithiocarbamates (broad-spectrum protectant molecules) reduces the risk of resistance development. The *N. clavispora*–cardamom host pathosystem further warrants investigation in the direction of exploring the occurrence, distribution and epidemiology as they may be disseminated inadvertently pan-cardamom-growing tracts through unrestricted transboundary movement of planting materials. Moreover, the biology and ecology need to be investigated as it has the potential for pathogenic transformation under conducive weather or stressed host conditions. *N. clavispora* is a commonly encountered endophytic species and reported as a plant pathogen in several plant genera (Keith et al. 2006; Wei et al., 2007; Espinoza et al., 2008), and hence, it is highly imperative to decipher pathogenic nature of this cryptic genus to gain further insights with respect to epidemiology and disease management perspectives. The present study constitutes first report on the association of *Neopestalotiopsis clavispora* with leaf blight disease of small cardamom.

5 | CONCLUSION

Among the foliar diseases of cardamom, leaf blight incited by *Colletotrichum* is considered as the major one, prevalent in all cardamom-cultivating tracts. In the present study, six symptomatological variants were delineated based on manifestation of foliar symptoms in cardamom genotypes, *viz*. Malabar, Mysore and Vazhukka and designated as SV 1 to SV 6. Among the symptomatological variants, SV 1, SV 2, SV 3 and SV 6 were more pronounced in Vazhukka, while SV 4 and SV 5 were prominent in Malabar. The pathogenic isolates from symptomatological variants were characterized with whitish colonies, fusiform, five-celled conidia with three median versicoloured cells, two terminal hyaline cells and measured 23.1–27.25 × 3.84–4.43 μm. The apical cells had two to three tubular, flexuous, unbranched appendages, whereas the basal appendage was single, tubular and unbranched. Based on conidial features and employing molecular methods with internal transcribed spacer rDNA region, partial β-tubulin, translation elongation factor 1 alpha and large subunit (28S) of the nrRNA genes, the pathogens were identified as *Neopestalotiopsis clavispora*. The pathogenicity test was performed on Malabar, Mysore and Vazhukka genotypes, and Koch's postulates were proved. The *in-vitro* interaction at three temperature regimes indicated that *N. clavispora* was inhibitory to *Colletotrichum gloeosporioides* at 10°C and 30°C. Carbendazim, propiconazole and carbendazim-mancozeb completely arrested hyphal growth of *N. clavispora* under *in-vitro* conditions. The present study constitutes first report on the association of *Neopestalotiopsis clavispora* with leaf blight disease of small cardamom.

6 | COMPLIANCE WITH ETHICAL STANDARDS

We hereby state that this manuscript has not been submitted to any other journals other than Journal of Phytopathology. The coauthors have no conflict of interests to declare, and no human participants or animals were used in the current research. All the authors provided the informed consent for the submission of the manuscript.

ACKNOWLEDGEMENT

The authors thank The Director, ICAR-Indian Institute of Spices Research, Kozhikode, for providing facilities and Indian Council of Agricultural Research, New Delhi, for financial support in the form of Outreach Programme on Diagnosis and Management of Leaf Spot Diseases in Field and Horticultural Crops.

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How to cite this article: Biju CN, Peeran MF, Gowri R. Identification and characterization of *Neopestalotiopsis clavispora* associated with leaf blight of small cardamom (*Elettaria cardamomum* Maton). *J Phytopathol*. 2018;166:532–546. https://doi.org/10.1111/jph.12715