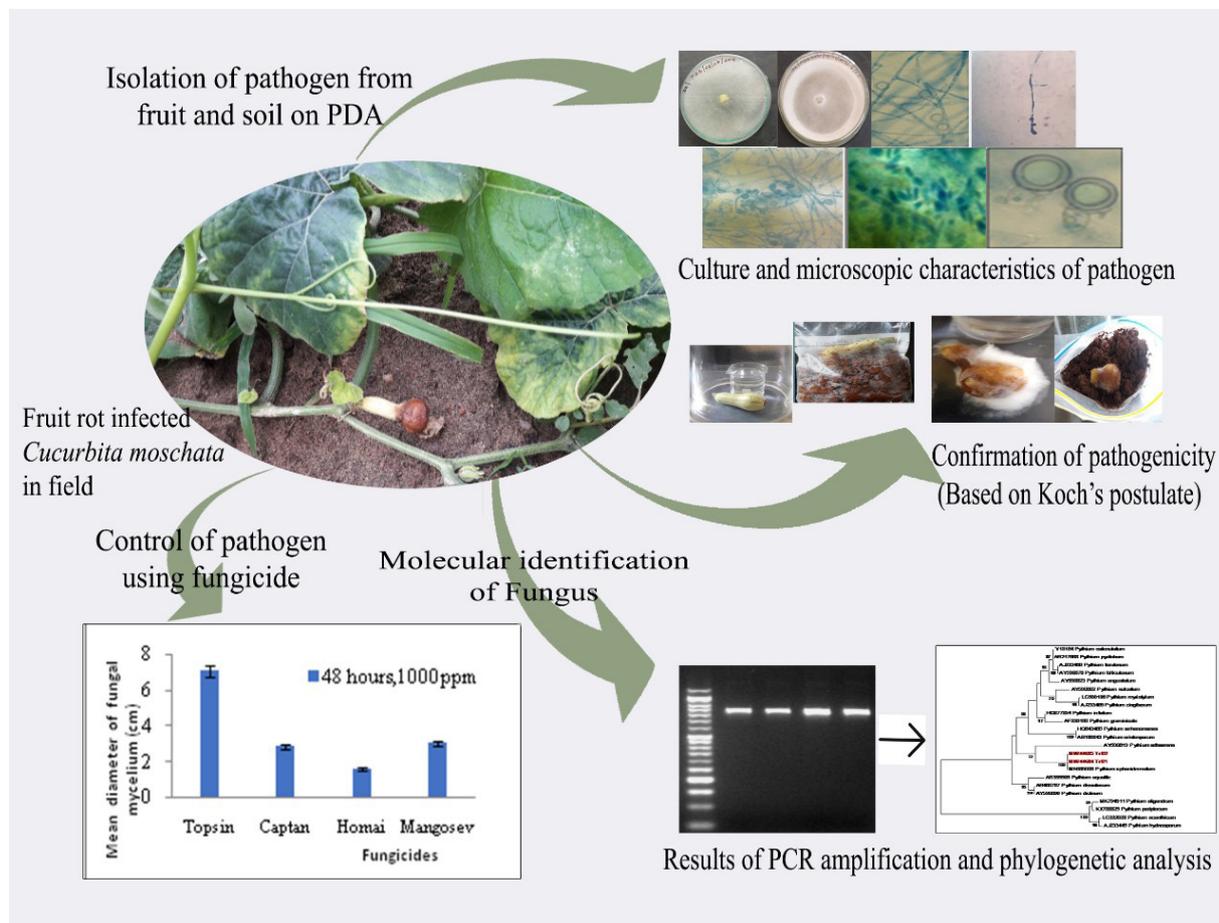# Fruit rot disease in butternut squash caused by *Pythium aphanidermatum* in Trincomalee district, Sri Lanka

P. Sevvel, D. Kugathasan and C.J. Emmanuel

Isolation of pathogen from fruit and soil on PDA

Culture and microscopic characteristics of pathogen

Fruit rot infected *Cucurbita moschata* in field

Confirmation of pathogenicity (Based on Koch's postulate)

Control of pathogen using fungicide

Molecular identification of Fungus

Results of PCR amplification and phylogenetic analysis

Highlights

- *Cucurbita moschata* fruit rot disease is caused by *Pythium aphanidermatum* in Trincomalee district
- The disease incidence was found to be varying between 44 – 66%
- Fungicides Homai or Captan (>1000 mg l^{-1}) can be used to reduce the pathogen growth

RESEARCH ARTICLE

Fruit rot disease in butternut squash caused by *Pythium aphanidermatum* in Trincomalee district, Sri Lanka

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Abstract: Fruit rot disease is a serious, emerging problem in butternut squash (*Cucurbita moschata*) cultivation in Northern and Eastern Provinces of Sri Lanka. Aim of the present study was to identify the causative agent of the fruit rot in the Trincomalee District and find a suitable fungicide to control the disease. Diseased fruit samples were collected randomly from four fields in Nilaveli, Morawewa, Kinniya and Thambalagamam. Fruit rots appeared brown colour, sunken and water-soaked spots which later turned black, enlarged rots with moldy appearance. The pathogen was isolated on Potato Dextrose Agar (PDA) medium. Cultural and morphological characteristics were studied. On PDA, cultures initially appeared white, cottony with heavy aerial mycelium which later became flat. Younger hyphae had swollen tips, while matured hyphae were hyaline, aseptate and dichotomously branched. Aplerotic oospores were surrounded by terminal oogonia. Zoospores arose from globose sporangia. There was no morphological variation among isolates, collected from the four different villages in the Trincomalee district. Internal transcribed spacer (ITS) region of the genome was PCR amplified. PCR products were subjected to DNA sequencing. Based on morphological features and analysis of ITS region of the genome, the pathogen was identified as *Pythium aphanidermatum*. Koch's postulates confirmed the pathogenicity of *P. aphanidermatum*. Four concentrations (10, 100, 1,000 and 10,000 ppm) of commercial fungicides (Captan 50 WP, Mancozeb 80 WP, Homai 80 WP and Topsin 70 WP) were tested against the isolate *in vitro*. Fungicides, namely Homai or Captan (>1000 mg l^{-1}), can be used to reduce the pathogen growth.

Keywords: Fruit rot, Butternut squash, *Pythium aphanidermatum.*

INTRODUCTION

Butternut squash (*Cucurbita moschata* Duch.) is a fruit vegetable of the family Cucurbitaceae. It is a fast-growing vine that creeps along the surface in a similar fashion as that of the other vegetables and fruits like pumpkin and cucumber of the family Cucurbitaceae. *C. moschata* is a highly polymorphic species which was first named and described by Duchesne in 1786 (Paris, 2000). Very old archaeological macro-remains reveal South American origin of *C. moschata* (Whitaker and Bemis, 1964). By the end of the 19th century, *C. moschata* had spread worldwide. It is the pre-dominant *Cucurbita* species in lowland tropical areas, but it is grown to a lesser extent in temperate regions (Andres, 2004).

The vegetable species belonging to Cucurbitaceae contribute significant amounts of vitamins and minerals to the human diet (Lucera *et al*., 2012). Butternut consumption decreases the risk of obesity, diabetes, heart disease and mortality but promotes complexion enhancement and increase in energy (Hanif *et al*., 2006). The potassium found in cucurbits prevents high blood pressure, reduce the risk of death from all types of stroke and cardiovascular diseases (Ananya and Raychaudhuri, 2010).

It is more tolerant to heat, insolation, and humidity than any other domesticated species of *Cucurbita*. Once plants are established, they are able to withstand not just wet conditions, but dry conditions as well (Ibrahim *et al*., 1996). Fruits of most commercial cultivars of *C. moschata* are harvested mature when the stalks are dry and can be stored for few months (Bailey, 1962).

The hard nature of butternut squash enables the crop to tolerate harsh environmental conditions to a certain extent and resistant to many pests of cucurbits (Bonjour *et al*., 1990). However, Cucurbitaceae members are susceptible to some fungal pathogens under field or storage conditions. Fungi such as *Fusarium culmorum*, *F. solani* and *Didymella bryoniae* have been reported to cause storage rot disease in butternut fruits (Hawthoren, 1988). Phytophthora blight, caused by *Phytophthora capsici*, can infect the entire range of cucurbits and is known to be a serious field disease. White mold, caused by the fungus *Sclerotinia sclerotiorum*, can cause losses in the field and in storage (Babadoost and Zitter, 1992).

Butternut squash is a popular vegetable in Sri Lanka and it has been cultivated in many parts of the country, especially in the dry zone. Based on recent field visits and meetings with farmers, it has been noted that many commercial farms are facing serious rotting disease symptoms in young butternut squash fruits in Northern and Eastern Provinces of Sri Lanka. The disease leads to severe yield reductions and, consequently, the farmers avoid growing butternut squash. To our knowledge there are no research reports or publications regarding this fruit disease from Sri Lanka. The present study is aimed to identify causative agent of the rotting disease in butternut squash

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cultivated in Trincomalee district, and to test the efficacy of common fungicides against the pathogen.

MATERIALS AND METHODS

Sample collection

Infected fruits and soil samples were collected from eight different commercial farms representing four regions, Nilaveli, Morawewa, Thambalagamam and Kinniya, in the Trincomalee district in Sri Lanka (Figure 1). Younger butternut squash fruits showing rot symptoms were carefully excised from infected plants. The samples of soil around the symptomatic fruits were also collected. All the samplings were done between June to December 2018.

Determination of disease incidence

To determine the disease incidence, total number butternut squash fruits and the number of symptomatic fruits in four fields in each region were counted. The disease incidence was determined using the equation,

% Disease Incidence= <u>Number of infected fruits</u>   x 100
                              Total number of fruits observed

Isolation of pathogen

Depending on the stage of disease development in fruits, the isolation technique varied slightly. In fruits with disease symptoms and cottony mycelial growth, a small portion of the mycelium was directly transferred on to PDA medium. The fruits that show early stages of symptom development were kept in a humid growth chamber for 1-2 days until the mycelium grows. Pieces of infected tissues cut from fruits were surface sterilized by dipping in 1% NaOCl for 2 min and, after rinsing three times in sterilized water, tissue pieces were placed on PDA medium incorporated with streptomycin (100 ppm). The plates were incubated at 27 °C with 12 h light (600 lux) in a growth chamber. Ten grams of a wet soil sample was suspended in 100 mL of sterilized water and the suspension was serially diluted tenfold. An aliquot (100 μL) from the 10^{-4} dilution was inoculated on PDA using spread plate technique. After 2-3 days of incubation, the colonies growing on the PDA plate were sub-cultured on fresh PDA medium.

Morphological identification

Morphological identification of the isolates was done based on characteristics of the isolates grown on PDA medium and microscopic features (Watanabe, 2002). Colony characteristics such as colour, form of mycelial growth (e.g. aerial, flat) and growth rate were measured. Vegetative and reproductive structures of the isolates were examined under light microscope (Olympus CX31, Japan). The diameter of hyphae, spores and other structures was measured using stage micrometer. Cotton blue was used for staining the mycelium.

Zoospore production was induced by growing the isolates on zoospore induction medium (one liter of the medium consisted K_2HPO_4 (43.55 g), KH_2PO_4 (34.03 g), $(NH_4)_2HPO_4$ (33.02 g), $MgCl_2.6H_2O$ (12.71 g) and $CaCl_2$ (59.19 g). Then about 2 cm long healthy grass leaves were collected and sterilized by autoclaving at 121 °C for 20 min. The leaf pieces were placed on the surface of two days old culture for 24 h and then they were immersed in the above prepared induction medium contained in a Falcon tube. The tube was incubated at room temperature for 24 h and the leaves were then stained and observed under high

Figure 1: Sample collection sites. Butternut squash fruit rot samples were collected from four different regions (N - Nilaveli, M - Morawewa, T - Thambalagamam and K - Kinniya) in Trincomalee district.

power of light microscope (Mendoza and Prendas, 1988).

Molecular identification

DNA extraction and PCR amplification

DNA was extracted from 3-day old cultures grown on PDA medium. DNA extraction was done using DNeasy Plant mini kit (Qiagen, Germany) as described in the manufacturer's guidelines. The ITS region of the genome was amplified by polymerase chain reaction (PCR) using primers ITS1 and ITS4 (White *et al*., 1990). The PCR reactions were carried out in a volume of 20 µL reaction mix containing 10 µL ready-made PCR mix (PCR Biosystems, UK), 1 µL of forward and reverse primers each (10 µM) and 1 µL DNA sample. Amplification was carried out in a thermal cycler (Techne Thermal Cycler- TC3000, UK) according to the following amplification program: an initial denaturation at 95 °C for 3 min followed by 35 cycles including denaturation at 95 °C for 30 s, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and the final extension step at 72 °C for 10 min. PCR products were electrophoresed on a 2 % agarose gel stained with ethidium bromide (10 mg/ml) and were viewed in a gel documentation system (Enduro GDS, Labnet, USA).

Sequencing and analysis of sequences

The PCR products of two samples, collected from two commercial farms situated about 25 Km distance in Trincomalee district, TrI01 (collected from Nilaveli) and TrI02 (collected from Kinniya), were sequenced using forward and reverse primers by automated Sanger sequencing service (Macrogen, Korea). The nucleotide sequences were deposited in GenBank® database.

Identity searches for the sequences were carried out by using the BLASTn program available in the NCBI. The sequence, which shows the highest sequence identity from non-redundant nucleotide database was detected for each isolate. Phylogenetically diverse 22 *Pythium* species were chosen based on previous studies (Le´vesque and de Cock, 2004; de Cock *et al*., 2014) and retrieved from GenBank database. All sequence data were aligned initially using ClustalW (Thompson *et al*., 1994) and then visually checked. The aligned data matrices were assessed to find the best-fit model of nucleotide substitution using jMODELTEST (Posada, 2008). The evolutionary history was inferred by using the Maximum Likelihood method based on the Hasegawa-Kishino-Yano model (Hasegawa *et al*., 1985). Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+*G*, parameter = 0.3300)). The tree was drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 23 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 709 positions in the final data set. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2016). Consensus trees were generated using the 50 % majority rule tree criteria.

Confirmation of pathogenicity

The pathogenicity of the isolates was tested following Koch's postulates. Young butternut squash fruits were inoculated with the pathogen using two different methods. In direct plant inoculation method, the young healthy fruits were surface disinfected by wiping with cotton wool wetted with 70% ethanol. After complete evaporation of ethanol, a hole with 5 mm diameter and about 3 to 5 mm depth was made on the fruit using sterile cork-borer. The mycelium, taken from a 5-day old pure culture with a sterile forceps, was transferred into the hole. The piece of tissue removed from the fruit was placed back in position and the hole was covered. The controls were treated with sterile distilled water instead of the mycelium. The inoculated and control fruits were incubated at room temperature in humid chambers for 4 to 7 days and development of disease symptoms was monitored through visual observation. This experiment was repeated 3 times and each time 3 replicates were maintained.

In the second method, the inoculum was prepared by flooding a 7 days old culture with sterilized saline water. The inoculum (20 ml) was mixed thoroughly with 10 g of sterilized soil, inside a transparent zip bag. Surface disinfected healthy, young squash fruits were placed into the zip bag such that the lower part of the fruit contacts the wet inoculated soil. In control experiment, the suspension was replaced by sterilized saline water. All the treatments were incubated at room temperature for 4 - 7 days and development of disease symptom was monitored. The pathogen was re-isolated on PDA using both methods. Morphological characters of the pathogen were compared with those of the initial isolate from diseased squash fruits.

In vitro evaluation of fungicide resistance

Fungicide-resistance of the isolate TrI01 was tested against four different fungicides, namely Captan (50 % WP), Topsin M (70 % WP), Homai (thiophanate-methyl 50% + thiram 30 % WP) and Mancozeb (80 % WP). Fungicide stock solutions were prepared at 100,000 ppm by dissolving the fungicides in sterilized water. The quantity of fungicide used for stock preparation was determined based on the percentage of active ingredient in each fungicide. The fungicide stocks were serially diluted tenfold and incorporated into PDA media. Finally, the PDA plates had four concentrations (10 ppm, 100 ppm, 1,000 ppm, 10,000 ppm) of the fungicide. The plates were inoculated with 10 mm diameter mycelial discs obtained from a 7-days old of culture. In control plates, sterile water was used instead of fungicide. Each treatment was repeated three times. The plates were incubated at 27 ºC with 12 h light in a growth chamber for 3 days. The mycelial growth was measured using a Vernier scale at 24 h, 36 h and 48h time periods.

RESULTS AND DISCUSSION

The fruit rot was exclusively observed on very young fruits of the butternut squash (Figure 2). The infected fruits exhibited brown color, water-soaked and sunken rotted

areas along the surface of fruit which was in direct contact with soil. In severe conditions, the infection spreads over most parts of the fruit and become enlarged, sunken and turned from brown to a black coloured rot. Under high humid conditions, white mycelial growth resembling tufts of cotton, was found on rotted areas.

The infected fruits were noticed in eight farms visited during the study, and the disease incidence was found to be varying between 44 – 66% (Figure 3). The disease incidence was significantly higher in Nilaveli and was 66%.

All the isolates showed similar morphological features. On PDA medium, over the first 5 to 8 days, heavy, cottony, pure white color, aerial mycelial growth was observed, but after 10 to 12 days the colony was observed as flattened mycelium (Figure 4). There was no colour change noticed in the colony to show spore production as in other plant pathogenic fungi. The isolates were found to be fast growing and within 36 h the mycelia completely covered the PDA plate.

The microscopic observation of young and mature hyphae revealed their hyaline, aseptate and dichotomously branched nature (Figure 5). Young mycelium was usually with swollen tips. Diameter of the hyphae did not vary (p>0.05) among the isolates. Mean diameter of young mycelium ranged between 3.18 μm to ٣٫٤١ μm while mean diameter of matured mycelium was between 4.85 μm to ٥٫٣٤ μm. Zoosporangia were terminal, inflated and globose. The zoosporangia released motile bi-flagella zoospores. The oogonia were terminal and lobose with thick-walled, aplerotic oospores. Antheridia were mostly intercalary, sometimes terminal, broadly sac shaped and arise as branch of the stalk of oogonium. Mean diameter of oospores of the isolates varied between 26.33 μm to 27.63 μm. The isolates obtained from diseased fruits and soil samples showed similar morphological features. Therefore, for the molecular identification, one isolate representing each region was selected. The PCR amplification with primers specific to ITS region yielded a single band with about 850 bp length in all four tested samples. Hence, only two isolates TrI01 (Nilaveli) and TrI02 (Kinniya) were selected for sequencing studies and confirmation of the causative agent.

Figure 2: Fruit rot symptoms in diseased butternut squash fruit under field condition. The fruit on the left is a healthy fruit, and two fruits on the right show characteristic rotting symptoms.
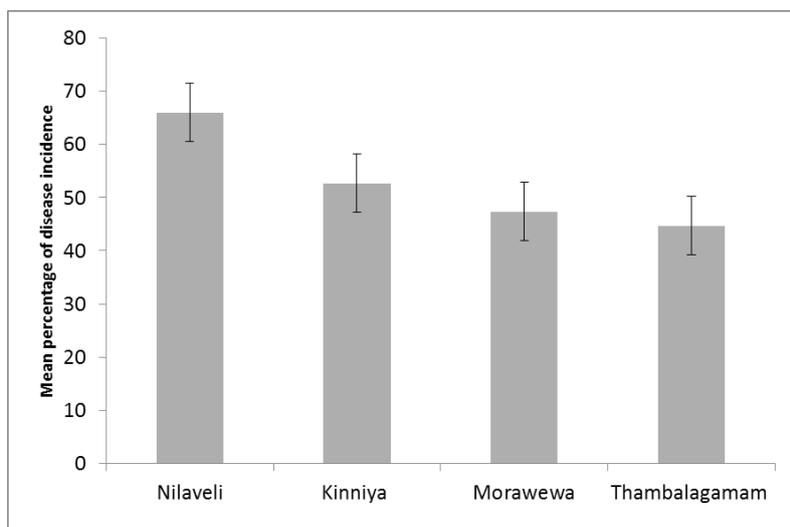

Figure 3: Incidence (%) of fruit rot disease in *C. moschata*. In each region disease incidence was determined in four different fields and the mean percentage of disease incidence is presented in this chart. Standard Error of mean = 5.48%.
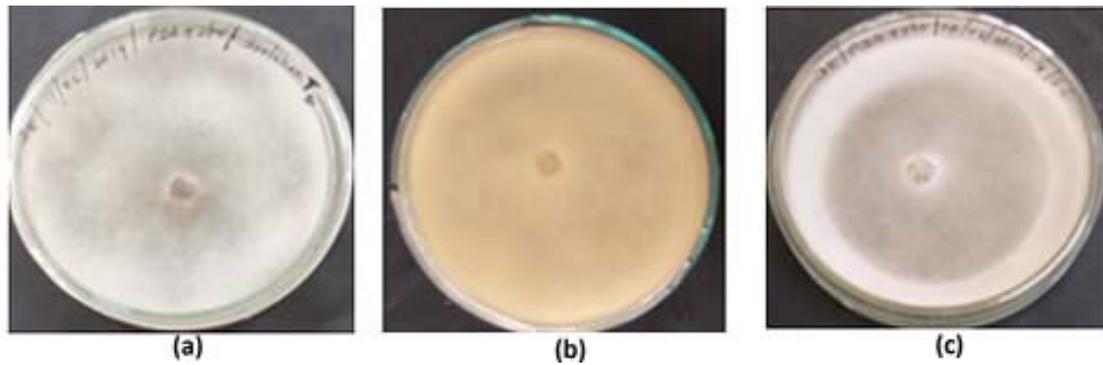
Figure 4: The pathogen isolates grown on PDA plate, (a) Surface view of a colony after 48 h, (b) under side of PDA plate after 48 h of inoculation, and (c) 14-days old culture.
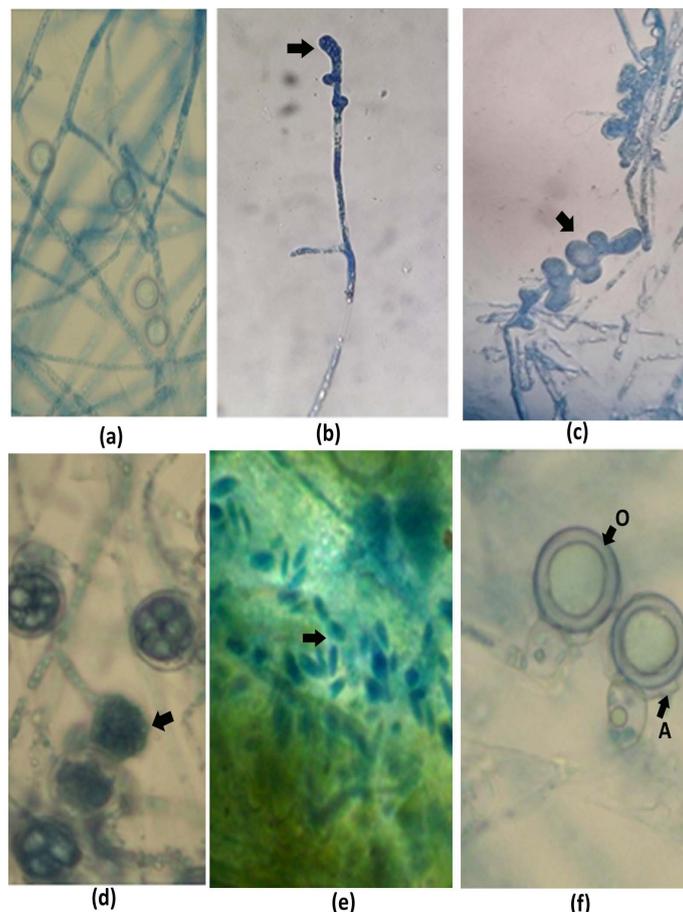

Figure 5: Microscopic images (10 x 40) of vegetative and reproductive structures of isolates. (a) Hyaline, aseptate, dichotomously branched hyphae, (b) The mycelium with swollen tips, (c) Lobose sporangium, (d) Zoospores within sporangium, (e) Zoospores in induction solution, (f) Oogonium and antheridium.

Sequencing of the two PCR products yielded sequences of 865nt and 864nt (TrI01 and TrI02, respectively). Sequence analysis showed that the two sequences have 18S ribosomal RNA gene (partial sequence); internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2 (complete sequence) and 28S ribosomal RNA gene (partial sequence). Both TrI01 and TrI02 showed highest sequence identity with isolates of *Pythium aphanidermatum* in BLASTn analysis. The sequences showed 99.88% (TrI01) and 99.65% (TrI02) sequence identity with *Pythium aphanidermatum* isolate FJTP01 (GenBank® accession KJ162355) reported from China. In phylogenetic analysis, based upon an alignment of selected ITS sequences of various *Pythium* species, both TrI01 and TrI02 clustered with *P. aphanidermatum*. The sequences were deposited in GenBank® database and accession numbers MN744684 and MN744685 were assigned to isolates TrI01 and TrI02, respectively.

The pathogenicity of the isolates, TrI01 and TrI02, was confirmed through Koch's postulates (Figure 8). The fruits inoculated directly and through soil inoculation showed disease symptoms. Diseased fruits were found covered with white coloured, cotton-like mycelium within 4 days
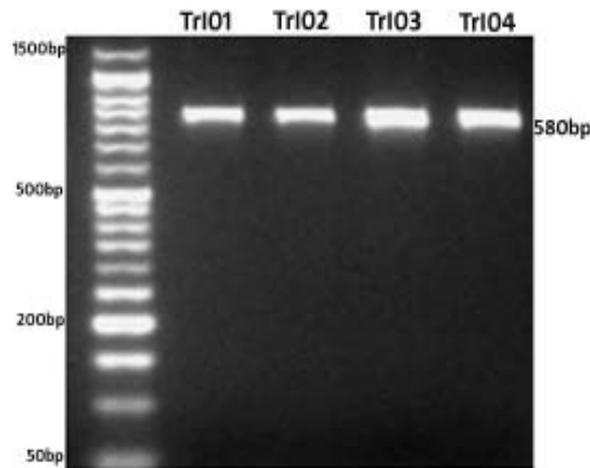
Figure 6: Gel-electrophoresis image obtained with four different samples, represent four different regions in Trincomalee district. Lane 1 = DNA marker. Lane 2-5 = PCR products obtained by the amplification of ITS region of the four isolates. The PCR product size is about 850 bp.
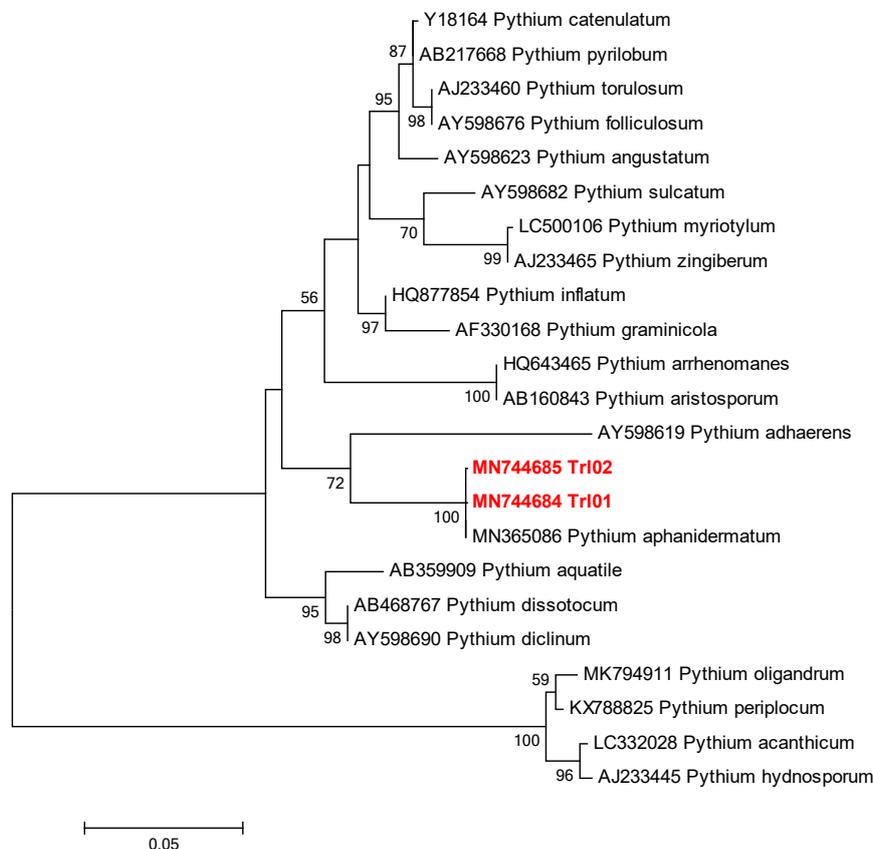

Figure 7: Molecular Phylogenetic analysis by Maximum Likelihood method. The percentage of trees in which the associated taxa clustered together is shown next to the branches. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

of inoculation. Re-isolation and microscopic observation of its morphology confirmed the pathogen.

Fungicide resistance assay revealed that the isolate TrI01 is resistant to all tested fungicides at low concentrations such as 10 ppm and 100 ppm. In the PDA medium, incorporated with 1000 ppm and 10,000 ppm concentrations, the extent of mycelial growth inhibition varied ($p<0.05$) among the fungicides tested in this study. There was no mycelial growth at 10,000 ppm of Captan or Homai.

The butternut cultivation is an emerging cultivation sector in Sri Lanka. But recent field visits to various parts of Northern and Eastern Provinces and meetings with farmers revealed a kind of rot disease in young fruits which reduced the yield in cultivation in the same regions of the country. The symptoms of the disease and the signs associated with rotting young fruits indicated *Pythium* cottony leak disease. The study was done to confirm the
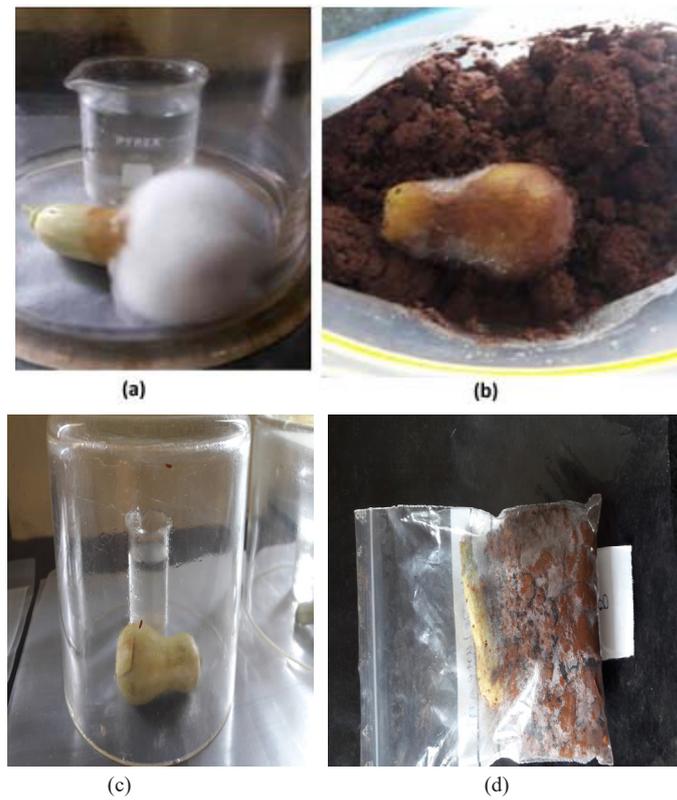
Figure 8: Koch's postulates to show pathogenicity of the isolates. The inoculated young fruit showed characteristic fruit rot symptom and cottony, pure white color mycelial growth. (a) fruit directly inoculated with the isolate, (b) fruit infected through soil inoculation, (c) and (d) respective controls.
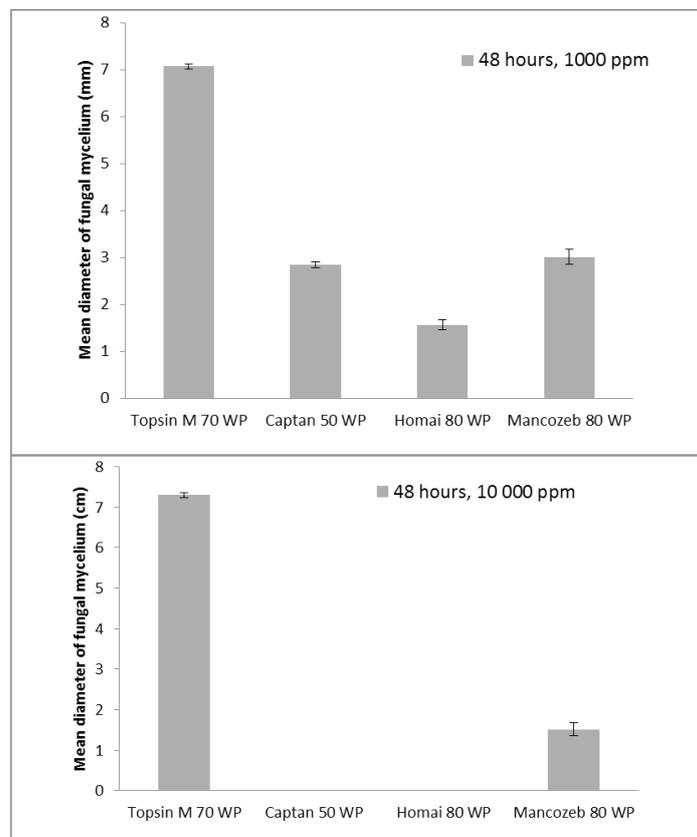

Figure 9: Fungicide sensitivity of *P. aphanidermatum* (isolate TrI01) against four different fungicides. Radial growth of mycelium in 1000 ppm fungicides after 48 h incubation.

disease and to identify the causative agent. The microscopic and molecular sequence-based identification showed the association of *P. aphanidermatum* with the disease. The Koch's postulates also confirmed the pathogenicity of *P. aphanidermatum*. To our knowledge this is the first report, which demonstrates butternut squash fruit rot disease caused by *P. aphanidermatum* in Sri Lanka.

Application of fungicides is the common approach to manage the disease in fields at commercial level. In the present study, four different common fungicides were tested against the pathogen, *P. aphanidermatum* under *in vitro* conditions. The study showed the requirements of > 1000 ppm of Captan (50 % WP) or Homai (thiophanate-methyl 50% + thiram 30 % WP) to control this pathogen.

CONCLUSIONS

The present study confirmed that the fruit rot disease in butternut squash is caused by soil borne *Pythium aphanidermatum* in Trincomalee district. I*n vitro* experiments have revealed the feasibility of managing the disease through the application of fungicides such as Captan and Homai. However, further studies are being conducted to identify the causative agents in other districts in Northern and Eastern provinces, and to test the possibilities to manage the disease through ecofriendly methods.

ACKNOWLEDGEMENTS

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest

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