PHENOTYPIC AND MOLECULAR CHARACTERISATION OF *Phytophthora* sp. INCITING LEAF FALL OF NUTMEG

By

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DECLARATION

I hereby declare that the thesis entitled **"Phenotypic and molecular characterisation of *Phytophthora* sp. inciting leaf fall of nutmeg"** is a bonafide record of research work done by me during the course of research and the thesis has not been previously formed the basis for the award to me any degree, diploma, fellowship or other similar title, of any other University or Society

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CERTIFICATE

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Introduction

1. INTRODUCTION

Nutmeg (*Myristica fragrans* Houtt), the two in one spice, is valued for its flavouring and medicinal properties It belongs to the family Myristicaceae The name *Myristica* is derived from the Greek word 'Myron' a sweet liquid distilled from the plant (Everett, 1981) Nutmeg is the seed kernel inside the fruit and mace is the covering (aril) on the kernel Both mace and kernel are used as condiment and medicine In India, it is cultivated in Kerala, Tamil Nadu, Karnataka, Goa, Maharashtra, North East India and Andamans Nutmeg plays a considerable role in India's agricultural export The total export of nutmeg/mace from India during 2011-12 was about 3620 tonnes worth of Rs 241 crore In India, Kerala is the major nutmeg producing state (Economic Reivew, 2012) Of the country's total production, Kerala accounts for 7000-8000 tonnes In Kerala, Ernakulum, Idukki, Thrissur and Kottayam are the major nutmeg growing districts (GOK, 2015) However, the productivity of nutmeg in our country is very low and it is mainly due to the non-adoption of scientific crop management practices and due to incidence of various diseases

In Kerala, the annual rainfall is always high varying from 3000- 6000 mm and about two - third of this rainfall is normally received during the South West monsoon periods of June to September Recently, a leaf fall disease caused by *Pytophthora* sp has become a serious problem in major nutmeg growing areas of Kerala during South West monsoon period This disease was first noticed at Mambra (Chalakkudy) in 2002 by KAU diagnostic team and later, wide spread occurrence was reported from Thrissur, Ernakulam, Kottayam and Idukki districts in 2011 The first authentic report of leaf fall of nutmeg due to *Phytophthora* sp was in 2012 from Kerala (Mathew and Beena, 2012) A severe outbreak of this disease has been reported from these districts in 2013 (Mathew and Miniraj, 2013) Experts and farmers's organizations estimated about 35 per cent dip in the total

yield during that year The impact of the attack can adversely affect the economy for the next three to four years if the problem is not tackled properly (Villatt, 2013)

Pytophthora is a ubiquitous and destructive pathogen of important cash crops of Kerala Various species of *Pytophthora* cause diseases such as foot rot of black pepper, capsule rot of cardamom, bean rot of vanilla, bud rot of coconut, mahali of arecanut, black pod of cocoa and abnormal leaf fall of rubber These diseases are very serious either causing heavy yield loss or death of the plant The genus, *Phytophthora* classified in oomycetes, includes more than 120 species that are mostly recognized worldwide as highly invasive plant pathogen After 2000, more than 70 new species of *Phytophthora* were identified internationally as plant pathogens occurring in crops and forest trees Nowadays, there is an increasing concern regarding the impact of *Phytophthora* on food, wood and fiber production worldwide

Considering the importance of nutmeg to the Kerala economy, it is necessary to study the emerging new disease of the crop and pathogen associated with it for the better disease management Therefore, the present study was undertaken giving emphasis on the following aspects

- Symptomatology of the disease
- Cultural, morphological and molecular characters of the pathogen
- Host range and cross infectivity of the pathogen
- Disease management

Review of literature

2 REVIEW OF LITERATURE

Dieback and fruit rot are the major diseases of nutmeg Recently a severe leaf fall disease caused by *Phytophthora* sp was noticed in Kerala during South West monsoon period The first authentic report of *Phytophthora* leaf fall of nutmeg was in 2012 from Kerala (Mathew and Beena 2012) A severe outbreak of this disease has been reported from major nutmeg growing areas of Thrissur, Ernakulam and Kottayam districts in 2013 (Mathew and Mmiraj, 2013)

2.1 Pathogen

Phytophthora, the 'plant destroyer', is one of the most destructive genera of plant pathogens in temperate and tropical regions, causing annual damages of billions of dollars (Erwin and Ribeiro, 1996) Nowadays, there is an increasing concern regarding the impact of *Phytophthora* on food and wood fibre production worldwide In 1996 less than 20% of *Phytophthora* species were known from forests and natural ecosystems (Brasier, 2009, Erwin and Riberio, 1996) Since 2000, over 50 new species have been described or are under description The majority of these new species are from forest ecosystems (Brasier, 2009)

The genus *Phytophthora* has been widely acknowledged as taxonomically 'difficult' (Brasier, 1983), as many of the characters used for species identification are plastic, highly influenced by environment, show overlap between species and have an unknown genetic basis However, currently new species are continually being formally described, with an estimated 200 to 600 species yet to be identified (Brasier, 2009) Through the increased use of molecular diagnostic techniques within the last 20 years, *Phytophthora* species are being revealed by large-scale environmental sampling in addition to the re examination of culture collections (Brasier, 2009, Jung and Burgess, 2009, Reeser *et al*, 2011) Additionally, new species have been identified through the increase in the international movement of plants, which has brought about new diseases not previously known in natural ecosystems and the nursery trade (Brasier, 2009)

In older classifications, the genus *Phytophthora* was placed in the family Pythiaceae, order Pernonsporales, class Oomycetes in the kingdom Fungi or Eumycota (Alexopoulos and Mins, 1979) In a more current classification system by Kirk *et al* (2008), the Eukaryotes have been divided into three kingdoms *Phytophthora* spp are placed in the kingdom Chromista under the phylum Oomycota

Genus *Phytophthora* is a historically important plant pathogen which was responsible for the Irish famine in 1845 (De Bary 1876) The type species, *P infestans*, destroyed Ireland's potato crop leading to a famine during the 19th century (Gregory, 1983 and Bourke, 1991) Even today, late blight is active and widespread and is responsible for high losses in potato production in many parts of the world (Duncan, 1999) The type species was characterized as having branched sporangiophores bearing sporangia which are deciduous and form zoospores within the sporangium, which germinate by releasing zoospores or by forming a germ tube

Phytophthora is one of the important pathogen causing diseases in several plantation and spices crops of Kerala *viz* coconut, arecanut, rubber, cocoa, black pepper, cardamom and vanilla *Phytophthora* sp attacking these crops were named differently by different workers Among them, *P palmivora P meadii P capsici* are the important ones Butler (1906) reported bud rot of coconut from India for the first time and the identity of the causal organism was proved as *P palmivora* and was accepted at international level in 1924 and 1926

Butler (1906) first reported fruit rot of arecanut caused by *Phytophthora* from South India and later in 1918, it was identified as *P arecae* Chowdappa *et al* (2003) opined that, *P meadii* was the main pathogen causing fruit rot of arecanut in India and there was no evidence of occurrence of *P arecae*

The earliest record of abnormal leaf fall disease of rubber in India was in 1910 from the estates at Palapilly in Thrissur district of Kerala and the organism was identified as *P meadii* (Mc Rae, 1918) Subsequently, it was reported from Sri

Lanka and Burma (Petch, 1921), and at present this is the most destructive disease of rubber in India

The occurrence of seedling blight of cocoa by *P palmivora* was first recorded by Chant (1957) from Nigeria which caused more than 70 per cent mortality of cocoa seedlings during high humid condition Black pod is another major disease of cocoa in this country which was also identified due to *Phytophthora* spp (Ramakrishnan and Thankappan, 1965) Chandramohanan *et al* (1979) identified the causative agent as *P palmivora* (Butl) Butl

The most destructive disease of black pepper, *Phytophthora* foot rot, which was first reported from Kerala by Samaraj and Jose (1966) and was identified *P palmivora* and later renamed as *P capsici* by Tsao and Alizedeh in 1985 Survey conducted at Calicut during 1982 84 and in Kannur (1985 - 86) showed 37 per cent and 94 per cent foot rot incidence amounting to a crop loss of 119 and 905 MT black pepper respectively (Balakrishnan *et al* , 1986, Anandaraj *et al* , 1988)

Menon *et al* (1972) reported *Azhukal* (inflorescence rot) disease of cardamom due to *Phytophthora* and was considered as the most important disease of cardamom next to Katte disease Bhai and Thomas (2000) observed severe rot disease of beans, leaves and stems of *Vanilla planifolia* during the south west monsoon season at Koothattukulam and surrounding areas in Ernakulam district of Kerala and the causative organism was identified as *P meadii* and is the first report of *Phytophthora* disease of vanilla in India

P ramorum is a recently emerged pathogen with a host range of more than 100 plant species This fungus causes sudden oak death on certain members of the oak family, and lead to the death of about one million trees in coastal forests in California The pathogen also causes, leaf blight or shoot blight on native plant species and horticultural nursery crops, and has plagued some nurseries in California, Oregon, Washington, British Columbia and in Europe (Rizzo and Garbelotto, 2003) It has been speculated that, tanoak were eventually removed completely from the landscape due to this disease

It is of great importance to prevent future introductions of new *Phytophthora* sp and to determine the species already present in natural ecosystems *Phytophthora* spp are found worldwide in many different ecological systems, where they can cause severe blight, damping off, or dieback of a wide range of plant species *Phytophthora* has come to the forefront of forest health in recent decades with the introduction of several non-native species into forests around the world that are causing disease on the landscape level (Holdenrieder, 2004)

2.2 Symptomatology

Symptoms of *Phytophthora* diseases depend on the specific *Phytophthora* sp and the host Mathew and Beena (2012) studied the detailed symptomatology of the leaf fall of nutmeg Symptoms first appeared as dark brown water soaked lesions on the midrib of the leaves which enlarged and spread along the lateral veins to leaf lamina resulting in blightening Petioles of the infected leaves showed black discoloration On young shoots, black lesions were observed which enlarged in size resulting in rotting and drying up of shoots from the tip downwards Leaf and stem infections resulted in extensive defoliation

Liyanage (1987) reported that, *P meadii* causes abnormal leaf fall and pod rot of as well as black strip in rubber and he observed that, symptoms were first seen on the immature green pods which are most susceptible Small lesions are indicated initially by pin head' black globules of latex, usually at the basal end of the pod, enlarge with continuous wet weather into brown water-soaked areas and covered with white mycelium Circular, brownish water-soaked lesions appear on the lamina with fine droplets of coagulated latex in concentric rings which later coalesce and cause rotting of the tissues Rajalakshmy (1995) described the symptoms of abnormal leaf fall of rubber as circular brownish water soaked lesions on the leaf lamina and led to premature defoliation, either green or after turning coppery red Lesion may develop on the midrib and leaf blades also A black lesion may also develop on the petiole with a drop of coagulated latex Heavy defoliation may lead to considerable loss of crop and die back of terminal twigs

The major symptoms produced by the attack of *P palmivora* include damping off, seedling blight, trunk canker, die back of twigs, blight and necrosis of leaf and petiole resulting in leaf fall and rotting of fruits, buds, flowers and calyx (Ramakrishnan and Seethulakshmi, 1956 , Chee, 1974) Prem (1995) described the symptoms of seedling blight of cocoa On young leaves, the disease developed as minute water soaked lesions, later turned to dark brown in colour and such lesions coalesced and resulted in blightening and defoliation

The first visible symptom of bud rot of coconut caused by *P palmivora* is the withering of the spindle marked by pale colour The spear leaf or spindle turns brown and bends over Basal tissues of the leaf rot quickly and can be easily separated from the crown Spindle withers and droops down one by one and the inner leaves also fall away, leaving only fully matured leaves in the crown A foul smell is emitted by the rotting tissue (Nambiar, 1994)

P citrophthora causes brown fruit rot, trunk gummosis, collar and root rot, leaf and shoot blight in citrus and the symptoms vary with host and growing condition (CMI, 1964) *P citrophthora* also produces symptoms like leaf chlorosis, wilt and dark brown water soaked lesion on leaves Graham (1998) also observed water soaked spots on citrus leaves infected with *P citrophthora* and these spots extended to the entire leaf leading to defoliation

Thankamma (1983) reported shoot rot and leaf fall caused by *P nicotianae var nicotianae* in cashew The disease is characterised by black lesion on stem with gum exudation and lesion get enlarged in size resulting in the collapse of the affected shoots and shriveling of older leaves Lesion first appeared on the midrib of the mature leaves, which later spread to the main lateral veins and leaf blade Leaf and stem infection resulted in extensive defoliation

Phytophthora rot in vanilla produced symptoms on leaves, stem and beans The symptom of the disease started as rotting in the form of dark brown water soaked patches on the petiole and lower portions of the youngest leaf which extended along the stem (Correll, 1953) Bhai and Thomas (2000) also studied the

symptomatology of *Phytophthora* bean rot in vanilla The symptoms first appeared as dark brown water-soaked lesions lead to rotting from the tip to the stalk The infected portions of beans became soft and dark brown and covered with white mycelial growth of the fungus

The leaf blight, caused by *P colocasiae* Rac is the most destructive disease of *Colocasia* The initial symptoms are the appearance of small round dark and roundish spots on leaves The lesions rapidly increase in size and cover large part of lamina (Oaka, 1990) The foot rot disease of black pepper caused by *P capsici*, showed the symptoms of collar rot, root rot and leaf disease as described and illustrated by Sarma *et al* (1991) Mammootty *et al* (1991) conducted detailed studies on symptoms of *Phytophthora* rot of pepper and infection was observed on leaves, stem and roots On leaves, infection started as water soaked lesions which later enlarged and become dark brown to black colour with smooth or fimbriate margins Severe infection resulted in defoliation The collar infection showed foliar yellowing, flaccidity of leaves, defoliation and breaking of stem at the nodal region and spike shedding Studies carried out by Anandaraj *et al* (1991) established that feeder root infection lead to collar rot and subsequent death of the vine

P ramorum causes two types of symptoms depending on the host Trunk cankers are seen on tanoaks while most shrubs and non woody plants show leaf spots which may be accompanied by shoot dieback Trunk cankers are the most damaging, and often lead to death (Rizzo *et al*, 2002) Davidson (2005) noticed dark brown lesions on *Rhododendron* leaves due to *P ramorum* infection which get enlarged and extended along the mid vein and showed water soaked appearance on the infected area

2.3 Characterisation of pathogen

2 3.1 Cultural characters on different media

There was some discussion on the usefulness of colony patterns for the identification of *Phytophthora* spp Waterhouse (1970) remarked that, patterns

should be considered as a taxonomic aid but Erwin and Ribeiro (1996) demonstrated variability of colony types among isolates of different species which makes this characteristic not useful for identification beyond supplementary purposes

The colony patterns like stellate pattern white cottony, floral pattern and uniform cotton wool like aerial mycelium and chrysanthemum like patterns were described by Brasier and Griffin (1979) on cocoa isolates of *P palmivora* They also observed sparse aerial mycelium with stellate and striated pattern with defined edge for *P palmivora* on carrot agar medium Kaosiri *et al* (1980) reported that, *P palmivora* isolate from different geographical origins could be grouped on the basis of five different colony patterns The difference in colony pattern was complemented by differences in their growth rates

Bhai and Sarma (2005) reported three distinct types of colony pattern in the isolates of *P meadii* from small cardamom *viz* uniform, cotton wool like aerial mycelium with vague lobbed pattern, stellate or petalloid pattern and lobed pattern Colony morphology of different *Phytophthora* species was studied by Widmer (2010) and compared by growing the isolates on carrot agar, Rye A agar and 20 per cent clarified V8 agar Some species showed very distinct patterns of growth on all the three media *P citricola* showed rosaceous patterns while *P syringae* was very distinctly stellate and *P heveae* showed stellate on V8 and Rye A agar and rosaceous on carrot agar Werres *et al* (2001) studied colony patterns of *P ramorum* on carrot piece agar, cornmeal agar, cherry decoction agar, V8 agar and oatmeal agar They observed that aerial mycelium was sparse or absent except on cherry decoction agar, where dense, appressed aerial mycelium was produced in weak rosette like patterns Pronounced concentric rings were formed on V8, and weak ones on carrot agar and cornmeal agar Shekhar *et al* (2011) observed four different growth patterns *viz*, cottony, petaloid, rosaceous and stellate for *P capsici* grown on PDA *P colocasiae* showed variable colony patterns from creeping whitish mycelium with slight zone of striations in V8 agar (Tsopmbeng, 2012)

Mounde *et al* (2012) described colony characters of *P nicotianae* and *P citrophthora* on amended corn meal agar (ACMA) Mycelium of *P nicotianae* mycelium was dense or loose rosette, with no pattern *P citrophthora* was characterized by a finely radiate, white rosette and slightly cottony colonies

2.3.2 Morphological characters

The morphology of an individual is the ultimate expression of its growth processes and final display of all its complex relationships with its normal habitat Identification of some *Phytophthora* species can be difficult due to lack of distinct morphological characters (Leonian, 1934, Brasier, 1971) In addition, morphological traits may overlap between species and such characters can be highly variable and dependent on growing conditions

A detailed description about the morphological characteristics of *P meadii* was given by several workers (Waterhouse, 1974, Stamps, 1985, Holliday, 1980, Rahman, 2014) According to them, sporangia are terminal or lateral, papillate, occasionally with two papillae, caducous, pedicel length of 10 - 20 µm in length, ellipsoid or elongated, obpyriform, occasionally spherical, often distorted into lobed or hourglass shapes Sporangia are 20 44 µm long x 16 29 µm wide with average size of 32 x 23 µm Chlamydospores are rare and ranged from 16 - 30 µm in diameter with average of 30 µm

Sansome *et al* (1975) and Brasier and Griffin (1979) observed abundant sporangia in carrot agar, corn meal agar, lima bean and oat meal agar and opinioned the carrot agar medium is the best medium for studying morphology of *Phytophthora* sp According to Al-Hedaithy and Tasao (1977), length of sporangial pedicel appears to be fixed for a species under normal conditions and is of high diagnostic value in identification of *Phytophthora* isolates Al Hedaithy and Tsao (1979) studied three *P palmivora* isolates on carrot agar, oat meal agar and V8-$CaC0_3$ and could not notice any difference in pedicel length in different media Size, shape and length to breadth ratio of sporangia were frequently considered as

important characteristics in identifying *Phytophthora* species (Ho, 1981, Waterhouse *et al* 1983 and Stamps *et al* 1990)

Dantanarayana *et al* (1984) studied the morphological characters of *Phytophthora* isolates from rubber and cocoa *viz P heveae, P arecae, P meadii, P botryosa, P megakarya* and *P palmivora* on lima bean agar *P meadii* differed from *P palmivora* in pedicel length, sporangial and sporangiophore morphology, chlamydospores frequency, size and colour and in oospore morphology and size Santhakumari (1987) isolated *Phytophthora* from bud rot affected coconut palms and observed spherical to elongated, ellipsoid, papillate and caducous sporangia with 30 7 µm length and 25 µm in breadth

Mammootty *et al* (1991) conducted detailed studies on the morphological characters of six black pepper isolates of *Phytophthora* on carrot agar medium According to them, the length of sporangia varied from 20 3 to 92 3 µm and breadth from 19 6 to 52 2 µm with the L/B ratio of 1 05 2 95 and pedicel length 3 5 - 3 8 µm Manohara and Sato (1992) studied the morphology and physiology of 43 *Phytophthora* isolates from black pepper in Indonesia Potato dextrose agar, corn meal agar, oatmeal agar and carrot agar were used to observe morphological characters Out of 43 isolate 42 had ellipsoid sporangia, which were markedly papillate, tapered at the base and caducous with long pedicel and the other one isolate was spherical sporangium with promment papilla Blaha *et al* (1994) worked on *Phytophthora* isolates from coconut plantations in Indonesia and Ivory Cost and observed, loose with less than 10% deciduous sporangia and L/B ratio of the isolates ranged from 1 2 1- 1 5 1

Several workers studied the morphological characteristics of *P citrophthora* and found out that, it produces sporangia in extremely variable shape *viz* ellipsoid, broadly ovoid, globose, limoniform or extremely distorted with prominent papillae often with two or more and sporangial length and breadth varied from 23 to 90 µm and 18 to 60 µm with L/B ratio of 1 3 1 1 8 1 In soil extract, sporangia borne singly or in very loose sympodia of two sporangia, often laterally

attached to sporangiophore, sporangiophore often with globose swellings at branching points and sporangia were non caducous (Mchau and Coffey , 1994), Akilli *et al* , 2012, Meitz Hopkins *et al* 2014)

On carrot agar, *P ramorum* produced sporangia singly or in clusters of 2 12 and arranged sympodially on long sporangiophores Sporangia were mostly ellipsoid, spindle shaped or elongated to ovoid and caducous with a short pedicel Sporangia were semipapillate (mostly 5-8 µm) which was less pronounced in young sporangia Sporangia were of 25 -97 µm length x 14 - 34 µm width with L/B ratio ranged from 1 7 to 2 0 (Werres *et al* 2001) Tsopmbeng (2012) described sporangial characters of *P colocasiae* as caducous, semi papillate with 40 to 70 µm long x 17 to 28 µm wide with an L/B ratio of 1 9 to 2 5

Sexuality is one of the complex area of *Phytophthora* biology All isolates of *Phytophthora* are potentially bisexual, that, they are able to produce both male and female sexual structures or gametangia (Galindo and Gallegly, 1960) However, only about half of the species of *Phytophthora* are homothallic and are able to produce oospores rapidly and abundantly in single culture The remaining species are heterothallic and produce gametangia only in response to chemical stimulation from an isolate of the opposite mating type (Ko, 1978 and Brasier, 1992)

The heterothallic species contain two mating types designated as A1 and A2 Oospores form after contact of the mycelia of A1 and A2 mating types Isolates of each type are bisexual and self-incompatible Relative degrees of maleness and femaleness occur within *P infestans* (Gallegly and Galindo, 1958) There was no consistent correlation between reproductive strategy (homothallic vs heterothallic) or antheridial attachment (paragynous vs amphigynous) with phylogenetic grouping The role of oospores in heterothallic species was not well understood, but evidences strongly indicated that the crossing of A1 and A2 mating type isolates could be a source of new races or biotypes when A1 and A2 mating types coexist

in nature, for example, *P infestans* on potato in the Toluca region of Mexico (Galindo and Gallegly, 1960)

Rosenbaum (1917) used six main criteria for the identification and separation of fungal species of which, the presence or absence and the size of the chlamydospores were the two important characters used Later, Waterhouse (1963) also used this property as a criterion in the identification of the species The presence, shape (globose, subglobose, elongate, obovate, obpyriform, distorted shapes, catenulate, radiate and clustered) and position (terminal and intercalary) are the features of chlamydospores which is useful in identification (Frank *et al*, 2012) Some workers used the absence of chlamydospores as one of the major criteria for separating or distinguishing *P capsici* from other morphologically similar species (Newhook *et al* 1978, Satour and Butler, 1968, Tucker, 1931, Waterhouse, 1963) Ribeiro (1978) observed that, *P meadii* did not produce chlamydospores in the medium and was considered as the distinguishing character of this fungus *P palmivora* produced adundant chlamydospores on potato dextrose agar and oat meal agar (Chandramohanan *et al*, 1979) Tsao (1991) reported globose to subglobose chlamydospore with a diameter of 28 to 29 µm for *P capsici* Mchau and Coffey (1994) noticed chlamydospore production by the majority of isolates of *P palmivora* on V8 juice agar with mean diameater of 30 20 µm

P tamoritm produced numerous chlamydospores on carrot piece agar, cornmeal agar, cherry decoction agar, V8 agar and oatmeal agar and were globose mostly thin-walled which formed intercalarlly and terminally, occasionally laterally and were the size of 20 91 µm (Werres *et al*, 2001) Misra (2011) observed single, globose terminal or intercalary chlamydospores for *P colocasia* with size of 17 to 38 µm

2 3 3 Molecular characterisation

Identifying *Phytophthora* species is accomplished using various morphological and molecular approaches (Oudemans and Coffey 1991, Brasier *et al* 1993, Erwin and Ribeiro 1996) In many cases, identification is difficult based

on morphological characters and the use of molecular genetics approaches is common (Cooke and Duncan 1997) The most frequently used genetic loci are based on the ribosomal RNA gene repeat (Cooke and Duncan 1997, Cooke *et al* 2000) The highly repetitive ribosomal RNA gene contains non translated sections known as internal transcribed spacers (ITS1 and ITS2) between the 18S and 28S genes 18S and 28S regions are highly conserved and are the basis for primers useful for amplifying ITS spacers in most *Pythium* and *Phytophthora* species (Cooke *et al* , 2000 Paul, 2001, Andre LeVesque and De Cock, 2004)

Through advancements in molecular techniques, the species of *Phytophthora* are currently organized into ten clades based on gene-wide phylogenetic analysis of two mitochondrial gene regions in addition to the nuclear internal transcribed spacer (ITS) region (Cooke and Duncan, 1997, Cooke *et al* , 2000, Martin and Tooley, 2003) New *Phytophthora* species are now characterized by this clade system, which has been validated using seven loci with 8700 nucleotide bases (Blair *et al* , 2008)

Lee and Taylor (1992) published ITS 1 and ITS 2 sequences of tropical *Phytophthora* species *P palmivora*, *P megakarya*, *P capsici*, *P citrophthora* and *P cinnamomi* and showed excellent resolution at the species level Trout *et al* in 1997 developed a rapid and accurate method for specific detection of *P infestans* by the construction of a new primer PINF The PINF primer will provide a valuable tool for the detection of *P infestans* in potatoes and tomatoes

The internal transcribed spacer regions (ITS 1 and ITS 2) of the ribosomal gene repeat from the *Phytophthora* species were amplified using the polymerase chain reaction and sequenced Sequences from *P cambivora* *P cinnamomi* *P citricola* *P cryptogea* *P drechsleri* *P fragariae* var *fragariae* *P fragariae* var *rubi* *P megasperma* var *megasperma* and *P nicotianae* were compared with published sequences and phylogenetic trees were produced The resultant grouping of species generally agreed with grouping established using classical morphological criteria, primarily sporangial morphology With improved technology, rapid

automatic sequencing of PCR amplified ITS regions is now possible and yields detailed information of relationship within the genus as well as allowing the design of species specific PCR primers for diagnostic purposes (Cooke and Duncan, 1997)

A molecular technique based on the internal transcribed spacer (ITS) region of ribosomal DNA was developed for the rapid identification of *Phytophthora* species DNA was isolated from cultures of *P capsici* from cocoa in Indonesia, *P nicotianae* and *P arecae* (*P palmivora*) from coconut in Indonesia, *P meadii* from rubber in Sri Lanka, Malaysia and India, and *P meadii* from arecanut in India ITS region from the mycelial extracts was amplified by polymerase chain reaction (PCR) using primers ITS 1 and ITS 4 The amplified product was digested with the restriction enzymes Hinf I, Msp I, Hae III and Rsa I Amplification with ITS 1 and ITS 4 yielded a PCR product of 860 bp for *P capsici*, 900 bp for *P arecae* and 920 bp for *P nicotianae* ITS - restriction fragment length polymorphism patterns of *P arecae*, *P capsici*, *P meadii* and *P nicotianae* significantly varied The isolates of the same species, however, showed identical banding patterns The results were almost similar irrespective of the enzyme used This method can be used as a taxonomic marker for pathogen identification and disease diagnosis (Chowdappa *et al* , 2003)

In a study conducted by Chowdappa *et al* (2003) regarding molecular discrimination of *P palmivora* isolates from cocoa and coconut and *P capsici* isolates of cocoa, black pepper and bell pepper were examined at the molecular level using ITS1 and ITS2 primers to amplify the internal transcribed spacer (ITS) regions of rRNA gene repeat yielded PCR products from the isolates contain a single band and size of the amplified product was 900 bp for *P palmivora* and 890 bp for *P capsici* ITS1 and ITS2 sequences of the *P palmivora* and *P capsici* have been published and detected excellent variation at the species level

ITS sequences of the nine species published by Lee and Taylor (1992) and Cooke *et al* (1996) confirm their utility in identifying species, determining natural groupings of species within the genus and gaining an understanding of their

evolution A clear grouping of species according to ITS sequence emergence was evident and it matched, to some degree, the classification based on type of papilla However, a separation of semi-papillate and papillate species was not evident and the papillate and semipapillate species found within groups I-IV (Waterhouse, 1970) was all grouped in the same clad, distinct from the clad consisting of the non papillate species from groups V VI Papilla type therefore may be a sound criterion for classifying *Phytophthora* sp

In an ITS sequence analysis (ITS 1 ITS-2) performed by Werres *et al* (2001) on 14 isolates of *P ramorum* showed that, all the isolates exhibited identical ITS sequences Their common ITS sequence was then compared to the ITS sequences of several *Phytophtora* species available from GenBank and *P ramorum* isolates were shown to be most closely related to *P lateralis*, from which they differed in the ITS-1 and ITS 2 regions by three and eight nucleotides, respectively They were unrelated to *P palmivora* and to the other species included as outgroups ITS region of 19 isolates of *P citrophthora* from different locations of Japan was amplified using PCR method and compared with other isolates of pathogen and revealed that, all isolates clustered one clade independent of host plants and geographic distribution, although there are some intra isolate variation (Jamal, 2007) Chowdappa (2011) observed that, the fungal isolates from arecanut, cardamom and rubber had identical ITS RFLP and AFLP patterns and that were different from *P palmivora* and *P arecae* isolates

2 4 Host range

Phytophthora spp cause disease in a large variety of dicotyledonous as well as monocotyledonous crops (Lamour *et al* , 2007) Species such as *P cinnamomi*, *P nicotianae* and *P cactorum* have a very broad host range, although others such as *P infestans* and *P sojae* are restricted to few host plants (Zentmyer, 1983) *Phytophthora* species tend to attack their hosts using enzymes which affect relatively unspecialised host chemical and mechanical resistance mechanisms (Brasier, 1983), whereas some host specific species are known to possess virulence genes which interact specifically, in a gene-for-gene system, with host resistance

genes (Thompson and Burdon, 1992) *P capsici* has wide host range including members of very diverse and phylogenetically distinct plant families such as solanaceous, cucurbits and leguminous crops (Erwin and Ribeiro, 1996)

Ashby (1927) described *P palmivora* as an omnivorous tropical fungal pathogen of worldwide distribution attacking a wide range of cultivated crops Bobr Tylingo (1954) listed the susceptible plants and stated that, *P palmivora* principally attacks cocoa, rubber, palms and various citrus fruits in addition to 56 species which are less severely affected Mowat (1963) observed that, *P palmivora* from cocoa did not infect leaves of pepper Pieris (1963) noticed that, the cocoa strain could infect rubber and *vice versa* Waterhouse (1963) reported that, the genus *Phytophthora* contains 39 species which affect a range of annual and perennial plants, shrubs and herbaceous flowering plants of many families Wide host range of *P citrophthora* with 83 genera in 51 families was reported by several workers (Gerlach, 1976, Orlikowski *et al*, 2001, Jamal, 2007) It was included in *Citrus* spp, strawberry, peach, *Rhododendron*, rubber etc Sarma *et al* (1987) studied the host range of *Phytophthora meadii* and reported rubber, cocoa, arecanut, cardamom and vanilla as hosts of the pathogen

P ramorum causal agent of sudden oak death disease has a wide natural host range The wide host range pathogen, *P ramorum*, was first identified in California from tanoak *(Lithocarpus densiflorus)* and coast live oak (*Quercus agrifolia*) (David and Rizzo, 1999) At present, species in over 70 different genera representing 33 different families of plants have been recorded as natural hosts (Werres *et al*, 2001 and Rizzo *et al*, 2002) *P ramorum* attacks plants in 12 families including Rosaceae (Different cultivar of *Rosa spp*) (Moralejo and Hernandez, 2002), Ericaceae (Rhododendron) (Garbelotto *et al* 2003), Myrtaceae (*Eucalyptus*) (Brasier *et al*, 2005), Fagaceae (*Quercus* spp and tanoak) (Denman *et al*, 2005) and Rutaceae (*Citrus limon* and *Citrus deliciosa*) (Moralejo *et al*, 2006)

Singh (2012) identified *Colocasia*, rubber, and black pepper as hosts of *P colocasiae* The host range of *P parsiana* a high-temperature tolerant newly described species was studied by Rafiee and Bamhashemi (2013) in southern Iran Among different plant species examined, almond was highly susceptible to different isolates

2.5 Cross infectivity

Cross inoculation studies with *Phytophthora* spp have been carried out by several workers Gadd (1927) showed that, cocoa and rubber isolates of *P palmivora* readily attacked wounded and unwounded cocoa pods, while coconut isolates failed to infect unwounded cocoa pods Rao (1930) observed the infection of *Phytophthora* sp of sandal, *Jatropha corcas* Linn and *Bryophyllum calycinum* to arecanut Loh (1970) observed that, an isolate of *P palmivora* from rubber produced typical lesions on detached leaves of black pepper

Radha and Joseph (1974) noticed that, *P palmivora* isolates of cardamom could infect rubber coconut and *vice versa* Thankamma (1983) stated that, *P nicotianae var nicotianae* isolates from pomegranate, black pepper and *Hibiscus* and *P meadii* from brinjal and *Artocarpus hirsuta* were pathogenic to rubber Das (1982) observed infection of *Phytophthora* isolates of pepper on arecanut, rubber, coconut and cardamom Azevedo and Silva (1986) moculated detached fruits of *Capsicum annum*, tomato, cucumber, melon and squash cultivars with an isolate of *P capsici* obtained from *Cucurbita moschata* and all were found susceptible to the pathogen Cross inoculation studies conducted with six isolates of *Phytophthora* from six different hosts like cocoa, pepper, arecanut, coconut, rubber and cardamom were positive and the symptom produced on the same hosts by different isolates was more or less identical (Mammootty, 1978)

Systematic studies on taxonomic complex of *Phytophthora* associated with black pod disease of cocoa revealed the occurrence of *P capsici* (Chowdappa *et al*, 1993) and *P citrophthora* (Chowdappa and Chandramohanan, 1996) besides *P palmivora* Thomidis *et al* (2002) studied the pathogenicity and virulence of 11

Phytophthora sp isolated from various hosts on apple and pear root stocks and only *P cactorum* and *P citricola* isolates were found pathogenic to these plants and isolates of *P cactorum* were most aggressive Abnormal leaf fall was reported to be caused by various species of *Phytophthora* including *P meadii*, *P botryosa*, *P capsici*, *P citrophthora* and *P nicotianae* (Sdoodee, 2004)

2.5 Disease management

Management of *Phytophthora* disease should be based on a sound understanding of the biology of the pathogen, including its modes of survival and dissemination, host range and the role of environment factors

2.5.1 Chemical control

Copper fungicides have been used since the early 1900's to control *Phytophthora* diseases The copper-based fungicides such as Bordeaux mixture, has been used since quite long time and is still the most effective chemical against the pathogen Other copper-based protectant fungicides include copper oxychloride, copper hydroxide, copper oxide, basic copper sulphate and copper ammonium carbonate, in which, Cu++ ion is the active ingredient against *Phytophthora* (Tollenaar 1958) Hishop (1963) reported that, many of the fungicides like Bordeaux mixture, Ziram, Captan and Panolil were toxic to *P palmivora* of cacao in the laboratory condition Cuprous oxide has consistently shown good control of *Phytophthora* diseases (Newhall, 1967)

According to Martin (1968), Okaisabor (1970) and Filani (1973), copper fungicides were highly fungicidal and inhibitory to zoospore germination of *P palmivora* under lab condition Filani (1976) noticed inhibition of *P palmivora* by cuprous oxide, copper sulphate, copper hydroxide and copper carbonate at all concentrations tested Effect of copper oxychloride in inhibiting the growth of *P palmivora* under *in vitro* condition was reported by Figueiredo and Lellis (1981) Reddy and Chandramohanan (1984) tabulated the relative efficacy of 24 fungicides against *P palmivora* of cocoa and observed that, fungicides Bordeaux mixture

(0 75%), Fytolan (1%) and Thiram (1%), Dithane M-45 (0 4%) and Captan (0 5%) completely inhibited the growth of the fungus in glucose nitrate agar medium

McGregor (1984) observed complete inhibition of *P capsici* and *P citrophthora* by Ridomil and Curzate at 25 ppm *In vitro* effect of Bordeaux mixture, copper oxychloride, copper hydroxide, mancozeb, metalaxyl and Antracol against foot rot pathogen of black pepper was reported by many workers (Turner, 1969, Mammootty, 1978, Ramachandran and Sarma, 1985) Prem (1995) noticed complete inhibition of *P palmivora* with Bordeaux mixture (1%), copper oxychloride (0 2%) and potassium posphonate (0 3%) under *in vitro* condition Platt (1998), Fernandez Northcote *et al* (2000) and Kirk *et al* (2005) noticed reduction in growth of *P infestans* by cuprous oxide, copper sulphate, copper hydroxide and copper carbonate at all concentrations tested under *in vitro* condition Shashidhara (2007) conducted *in vitro* evaluation of Akomin and Ridomil at 0 1, 0 2 and 0 3 per cent concentrations and were found effective against *P capsici* causing foot rot in black pepper Effectiveness of iprovalicarb fungicide against *Phytophthora* sp was reported by Thind (2011) Sharadraj and Chandramohanan (2014) reported cent per cent inhibition of *P palmivora* with cymoxanil 8% + mancozeb 64% (2 5g/l)

Many studies were conducted to investigate the effectiveness of the chemicals against *Phytophthora* diseases in field condition It was observed that, Bordeaux mixture, copper oxychloride, cuprous oxide, copper hydroxide, difolatan and organic tin compound were effective in reducing the black pod rot of cocoa (Gorenz, 1970, Gregory, 1974 and Thorald, 1975) Chandramohanan (1983) found that, drenching the cocoa seedlings with Bordeaux mixture or copper oxychloride just before the onset of monsoon and thereafter at frequent intervals resulted in good control of seedling die-back caused by *P palmivora*

The efficacy of Bordeaux mixture as soil drench and spray for the control of *Phytophthora* disease of black pepper was well established by Mammootty *et al* (1988) Sarma *et al* (1987) noted that, spraying the cuttings in the black pepper nursery with Bordeaux mixture, copper oxychloride or prophylactic spray with

Ridomil and Ziram at monthly intervals provided good control of *Phytophthora* rot in nursery Reduction in the incidence of foot rot of black pepper with Bordeaux mixture, pasting, spraying and drenching was noticed by Nair and Sarma (1993)

Copper oxychloride in mineral oil is extensively used in India, Malaysia and Sri Lanka as a preventive spray in the management of *Phytophthora* leaf fall of rubber (Jayasinghe and Jayaratne 1996) Metalaxyl, oxadixyl, captafol, folpet or mancozeb are recommended for panel treatment to control black stripe (Tan 1983, Jayatissa *et al*, 1994, Jacob *et al*, 1995) According to Jacob *et al* (1995) 0 8 per cent phosphorous acid provided effective and economic protection of tapping panels of rubber trees from black stripe disease when applied at weekly intervals

Thomas *et al* (1989) noticed effective control of *P meadii* on *Elettaria cardamomum* with 0 3 per cent Aliette 80WP and 1% Bordeaux mixture Spraying of one per cent Bordeaux mixture, 0 3 per cent potassium phosphonate and drenching soil with 0 2 per cent copper oxychloride were effective against azhukal disease of cardamom (Spices Board, 1999) Aerial spraying and soil drenching of potassium phosphonate (Akomin-40) gave maximum reduction of foliar and root infection of black pepper caused by *P capsici* (Veena and Sarma, 2000) They also noticed that, sporulation of *P capsici* of black pepper was the most sensitive stage to potassium phosphonate and that the mycelial growth was least affected

Guest (2002) reported that, potassium phosphonate not only protects cocoa and coconut from *Phytophthora* infection but also, increased the tree survival and yields and had an important role in integrated disease management strategies Bhai and Thomas (2000) observed that, spraying of 1% Bordeaux mixture twice and drenching with 0 25 per cent copper oxychloride were effective in controlling *Phytophthora* rot in vanilla Bhai and Sarma (2005) reported that, spraying of one per cent potassium phosphonate soon after the initiation of monsoon showers reduced capsule rot of cardamom caused by *P meadii* and soil borne inoculum Boughalleb (2006) reported the effectiveness of cymoxanil + mancozeb and

iprovalicarb +propineb against *P cactorum* affecting apple trees and their ability to absorb and translocated by roots of apple tree Similarly Mushrif *et al* (2011) also observed the effectiveness of cymoxanil + mancozeb, in the management of patch canker of rubber caused by the *Phytophthora* sp under *in vitro* and field conditions

According to Foster and Hausbeck (2010), copper hydroxide (0 2%) provided greater control against *Phytophthora* diseases, when applied as spray and drench Likewise, Garbelotto *et al* (2010) suggested copper hydroxide as the most effective fungicide for controlling *P ramorum*

2.5 2 Biological control

Biological control strategy was proposed half a century ago, as a result of several negative effects of the increased use of agro-chemicals on the environment and human health Sanford and Broadfoot (1931) were the pioneers to introduce the term biological control in Plant Pathology and conducted the first experiment on biological control of plant pathogens with antagonists Natarajan and Manibhushanarao (1996) reported that, use of fungal antagonists against fungal pathogens had gained considerable attention and appears to be promising as a viable supplement to chemical control *Coniothyrium Gliocladium Trichoderma Latesaria, Sporodesmium Aspergillus* and *Fusarium* and several bacteria and actinomycetes are known for their potential biocontrol activities against pathogens including several species of *Phytophthora* (Malajezuk, 1983, Adams, 1990, Naik and Sen, 1992)

Effect of *Trichoderma* against *Phytophthora* pathogen have been reported by many workers Liu and Baker (1980) reported that, the genus *Trichoderma* is a potential biocontrol agent against plant pathogenic fungi The large scale use of *Trichoderma* as a biofungicide in control of plant disease was reported by Cates (1990)

Antibiotics produced by *Trichoderma* spp have long been reported to be involved in biocontrol activities (Weindling, 1934) Pyke and Dietz (1966)

described dennadine, a major volatile antibiotic produced by *Trichoderma* Dennis and Webster (1971) stated that, the inhibitory action of antagonists against the pathogen *in vitro* might be due to the production of inhibitory volatile metabolites The mechanisms by which, *Trichoderma* spp suppress the diseases include competitive saprophytic ability, antibiotic production, direct parasitism and lysis (Ayers and Adams, 1981 and Bell *et al* 1982)

Trichoderma sp was observed to inhibit the growth of *P meadii* and cause lysis of oospores (Vanitha *et al*, 1994) Bhai (2000) observed overgrowth of *Trichoderma* spp on *Phytophthora* and parasitized the hyphae when both were grown on agar media She also noticed hyphal lysis, penetration and coiling of the parasite besides the production of volatile compounds Vijayaraghavan (2003) noticed complete inhibition of *P capsici*, with *Trichoderma* spp under *in vitro* condition Hernandez *et al* (2011) reported the antagonistic effects of thirty one *Trichoderma* strains isolated from different regions of Mexico against *P capsici* and volatile compounds were observed in 24 *Trichoderma* strains which showed inhibitory effect on *P capsici* in a range of 4 3 to 48 8 per cent

Galindo (1992) observed that, *Phytophthora* pod rot infection of cocoa with *T harzianum T viride* and *Gliocladium virens* have been effective in the control of *Phytophthora* sp causing cotton root disease (Heller and Hedtrich, 1994) The ability of *T harzianum* to control the rotting of hot pepper (*Capsicum annuum*) caused by *P capsici* was reported by Ahmed *et al* (1999) Bhai *et al* (1999) noticed the effectiveness of *Trichoderma* spp for management of the azhukal disease in cardamom caused by *P meadii* Joe (2000) found that, *Trichoderma* mixed with compost was effective in controlling *Phytophthora* root rot in cardamom and quick wilt in black pepper Chowdappa and Chandramohanan (1997) noticed the effectiveness of *T harzianum* as a potential biocontrol agent in the management of pod rot disease of cocoa

Jayasuriya *et al* (2002) suggested a possibility of increasing the resistance towards leaf disease caused by *P meadii* in susceptible clones of rubber by introducing *Trichoderma* as a biocontrol agent Vijayan (2004) obtained good disease

control of *P meadii* by the application of *T harzianum* Mathew (2008) reported the efficacy of *T viride* and *T pseudokoningii* of black pepper and *T harzianum* of vanilla against *P capsici* and *P meadii* under *in vitro* and *in planta* condition

Galindo (1992) noticed that, *P fluorescens* isolated from cocoa pods were antagonistic to *P palmivora* under field condition Myatt *et al* (1993) opined that, *Pseudomonas cepacia* was effective in inhibiting *Phytophthora megasperma f sp medicaginis,* the incitant of root rot of chickpea both under laboratory and field conditions Sarma *et al* (1996) and Diby *et al* (2005) noticed that, fluorescent Pseudomonads were effective in checking the growth of *P capsici* and in suppressing the expression of foot rot symptoms in black pepper under controlled conditions Tehrani and Omatie (1999) noted the efficiency of fluorescent *Pseudomonas* spp against soil borne fungal pathogens

Rubio *et al* (2000) noticed 74 per cent inhibition of *P infestans* with *P fluorescens* They also reported a clear zone of inhibition in the dual plate which is suggestive of production of antagonistic metabolites by *P fluorescens* Numerous mode of action have been reported for the antagonistic effects of *P fluorescens* in controlling diseases, which include the production of volatile and nonvolatile metabolites, competition for nutrients, production of enzymes and induced systemic resistance (Nandakumar *et al* , 2001, Ding 2010 , Vijayan, 2011, Ambuse, 2015) Lee *et al* (2003) extracted an antibiotic aerugine from the culture filtrates of *P fluorescens* Treatment which exhibited high protective activity against the development of *Phytophthora* disease in pepper and anthracnose in cucumber

Kurian (2011) observed reduction in severity of seedling blight of cocoa by the application of endophytic *P fluorescens* Stephan *et al* (2011) stated that, the combined application of *Trichoderma* spp and *P fluorescens* was more effective than in distinct application in controlling late blight of potato Koche *et al* (2013) tested antifungal activity of thirty isolates of *P fluorescens* obtained from citrus rhizosphere against *Phytophthora* spp and observed 40 per cent inhibition of the pathogen

Materials and methods

3. MATERIALS AND METHODS

The present study on "Phenotypic and molecular characterisation of *Phytophthora* sp inciting leaf fall of nutmeg" was carried out at the Department of Plant Pathology and Agricultural Microbiology, College of Horticulture, Vellanikkara during 2014- 2015 The main experiments except molecular work were conducted during the months of June to September, the ideal period for *Phytophthora* diseases

3 1 Collection of samples

Diseased samples were collected from different locations of Thrissur, Ernakulam and Kottayam districts of Kerala Samples were brought to the laboratory, washed under tap water, wiped with blotting paper, air dried and used for isolation

3.2 Isolation of the pathogen

The pathogen was isolated from infected nutmeg leaves and fruits, showing typical symptom The infected plant parts were cut into small bits of 5 mm size and surface sterilized with one per cent sodium hypochlorite solution for one minute and washed in three changes of sterile water The bits were then transferred aseptically to sterile Petri dishes containing carrot agar medium (Appendix I) Inoculated dishes were incubated at 22±1^{0}C for 2 5 days and observed for the fungal growth arising from the plant tissue Fungal growth on the medium was subcultured and purified by hyphal tip method Pure cultures of different isolates of the fungus were maintained on carrot agar slants and in sterile water and stored at 22±1^{0}C for the subsequent use

The isolates were given identification number, representing the name of pathogen, place of collection, plant part used for isolation and the Arabic numerals in serial order

3.3 Pathogenicity test

A preliminary study on pathogenicity was conducted with Mambra isolate (the first isolate obtained in the present study) of the pathogen on detached nutmeg twigs kept in conical flask containing sterile water, under lab condition Inoculation was done on leaves, petiole, stem and fruits of nutmeg with culture disc and zoospore suspension of the pathogen after giving pinprick injury The pathogen was reisolated from all infected parts and compared with the original culture

3.3 1 Pathogenicity of different isolates

Pathogenicity of different isolates of the pathogen was carried out on three month old nutmeg seedlings under *in vivo* condition adopting both inoculation methods as detailed below

a. Culture disc inoculation

Eight millimetre mycelial disc of the isolates from five day old culture was inoculated on the midrib region of upper and lower surfaces of the leaves, with and without injury Pin prick injury was given on the area near to midrib with four at the corners and one at the midrib Inoculated area was covered with moistened cotton

b. Zoospore inoculation

Zoospore suspension having concentration of 10^6spores/ml of water was prepared and drop of Tween-20 was added as spreading agent The seedlings were sprayed with 10 ml of zoospore suspension of different isolates on lower leaf surface after giving ten uniform pin pricks Seedlings inoculated with sterile water served as control

Five leaves in each plant were moculated and three plants were kept for each isolate The inoculated plants were covered with moistened polythene bags to

provide humidity and observed daily for the symptom appearance The pathogen was reisolated and compared with the original cultures

Based on the results, inoculation with culture disc on the lower surface of the leaves with pin prick was adopted in all other experiments unless otherwise mentioned

3 4 Symptomatology

Symptomatology of the disease on leaves, shoots and fruits was studied under both natural and artificial conditions Symptomatology under natural condition was studied during South West monsoon, the peak period of disease occurrence

For studying the symptomatology of the disease under artificial condition, 8mm culture disc of the 18 *Phytophthora* isolates were inoculated separately on leaves and shoots of the seedlings and on detached half matured fruits For root inoculation, seven day old inoculum grown in sterilized carrot bits were applied @ 10g/ plant, to the collar region of the seedlings after wounding These plants were covered with the moistened polythene bags and kept for symptom appearance

3 5 Studies on the virulence of various *Phytophthora* isolates

Eighteen *Phytophthora* isolates obtained from different locations were inoculated on detached leaves and also on nutmeg seedlings, to study the variations in their virulence Leaves of uniform size were inoculated with culture disc on both leaf surfaces with injury Three replications were maintained for each isolate Observations on the lesion size and days to leaf fall were recorded daily for seven days after inoculation

Virulence of the pathogen was categorised based on discrete statistics Based on mean and standard deviation of leaf area infected and per cent leaf fall, the isolates were classified into three groups, of which group 3 considered as highly virulent as it showed maximum infected area, higher per cent leaf fall which are indicators of high virulence and group 1 was least virulent While considering the

days to leaf fall as a measure of virulence, group 1 was found highly virulent as it exhibited early leaf fall and group 3 as least virulent Whereas, the group 2 represented moderately virulent isolates

The most virulent isolate identified was used for the host range and disease management studies

3.6 Cultural and morphological characteristics of different isolates of the pathogen

Cultural and morphological characters of 18 isolates of the pathogen were studied on different media *viz* carrot agar, potato dextrose agar, oat meal agar, coconut water agar, and V8 juice agar (Appendix I) at 22±1^{0}C

3.7 Cultural characters of different isolates of the pathogen

Eight mm sized culture disc from five day old culture of various isolates of the pathogen were placed at the center of the mediated plates and incubated at 22±1^{0}C Three replications were kept for each isolate and for each medium Observations on colony characters like colour, growth pattern and growth rate in different media were recorded at 24 h interval till full growth attained in the plates

3.7 Morphological characters

Morphological characters of 18 isolates were studied by slide culture method Morphological characters *viz* type of mycelium, type of sporangiophore, sporangial shape, size, L/B ratio and pedicel length were observed under research microscope Chlamydosopres and sexual structures on different media were examined by preparing slides from two week old cultures Microphotographs and measurements were taken using ultrascope

3 8 Molecular characterıstıcs

3 8.1 Isolatıon of DNA

Phytophthora ısolates were grown separately ın 100 ml of sterılc carrot dextrose broth at 25°C and mycelıum was harvested by fıltratıon after three days of ıncubatıon DNA was ısolated usıng the GenElute Plant Genomıc DNA Mmıprep Kıt (SIGMA) accordmg to the manufacterer's ınstructıons (Anonymous, 2011), and was eluted wıth 100 µl elutıon solutıon DNA was stored at – 20°C for further utılızatıon

3.8 2 Assessıng the quality of DNA

3.8.2 1 Agarose gel electrophorosis

Agarose gel electrophorosıs was performed based on the method descrıbed by Sambrook *et al* (1989) to check the qualıty of DNA and also to separate the amplıfied products

Procedure

1 1X TAE buffer was prepared from 50X TAE stock solutıon
2 Agarose (1 0 per cent (w/v) for genomıc DNA) was weıghed and added to 1X TAE It was boıled tıll the agarose dıssolved completely and then cooled to lukewarm temperature
3 Ethıdıum bromıde was added to a fınal concentratıon of 0 5µg/ml as an ıntercalatıng dye of DNA and mıxed well
4 The open ends of the gel castıng tray were scaled wıth a cellophane tape and placed on a perfectly horızontal levelled platform
5 The comb was placed properly and molten agarose was poured ınto the tray, allowıng ıt to solıdıfy
6 After the gel was completely set (15 to 20 mınutes at room temperature), the comb and cellophane tape were carefully removed
7 The gel was placed ın the electrophoresıs tank wıth the wells near the cathode and submerged ın 1X TAE buffer to a depth of 1cm

8 A piece of cellophane tape was pressed on a solid surface and 1µl 6X loading dye was added was dispensed in small quantity on the tape A quantity of 2 to 5µl of DNA was added to each slot, mixed well by pipetting in and out for 2 to 3 times Then the mixture was loaded in the wells, with the help of micropipette Gene Ruler 1kb was used as the DNA lader

9 The cathode and anode were connected to the power pack and the gel was run at a constant voltage of 100 volts

10 The power was turned off when the tracking dye reached at about 3cm from the anode end

3.8 2.2 Gel documentation

The DNA bands separated by electrophoresis were viewed and photographed using gel documentation imaging system

3.8.3 Reconstitution of primers

The lyophilized fresh primers were reconstituted by dissolving the primers with the 10X volume as it concentration by distilled water For PCR reaction the stock was diluted into 1 9 (1 µl primer 9 µl distilled water)

3.8.4. Amplification of the ribosomal internal transcribed spacer (ITS) genes

From eluted DNA, 5 µl were taken in eppendoff tube and kept at 98^0C for two minutes to denature After a brief centrifugation, 2 µl was taken and used as a template for amplification of 18S rDNA sequence The primer sequence was first discovered by Lee and Taylor (1992) and the primer details are given below

Primer details	Sequence 5'-3'	Length (Base pairs)
ITS1-Forward primer	TCCGTAGGTGAACCTGCGG	20
ITS4- Reverse primer	TCCTCCGCTTATTGATATGC	20

Polymerase chain reaction (PCR) was carried out in Eppendorf Master Cycler The composition of the reaction mixture for PCR is as follows

Component	Per reaction volume required
Template	2µl
Emerald Amp GT PCR Master Mix	12 5µl
Forward primer	0 5 µl
Reverse primer	0 5 µl
Distilled water	9 5 µl
Total	25 µl

A momentary spin was given to mix completely all reagents and set in thermal cycler for amplification The details of the thermal cycler programme are as follows

No	Steps	Temperature (^{0}C)	Time (Mm)
1	Initial denaturation	94°C	4 00
2	Denaturation	94°C	1 00
3	Annealing	55°C	1 00
4	Primer extension	72°C	1 50
5	Step 2-4	34 cycles	-
6	Final extension	72°C	5 00

3 8.5 Sequencing of the product

The product was purified and sequenced at Scigenome, Cochin, using ITS-1 forward and ITS 4 reverse primers

3 8 6 Nucleotide sequence analysis

The blast programme (http //www ncbi nlm nhm gov/blast/) was used to find out the homology of the nucleotide sequences The National Centre for Biotechnology Information (NCBI) accession sharing maximum homology with the query sequence was used to identify the isolate Dendrogram and phylogenetic analysis was carried out using the programme Clustal W

3.9 Host range of the pathogen

Four plantation crops and spices, two ornamental plants and one each of medicinal plant, tuber and fruit crop were artificially inoculated with *Phytophthora* isolate of nutmeg to find out its host range (Table 1) Inoculation was done with zoospore suspension (10^6spores ml^{-1}) / culture disc of the pathogen and the pathogen was reisolated from the inoculated leaves showing symptoms

3.9.1 Artificial inoculation on detached leaves under lab condition

3 9.1.1 Zoospore inoculation

Detached leaves of selected host plants were surface sterilized with one per cent sodium hypochlorite solution and washed with three changes of sterile water and wiped with blotting paper These leaves were kept separately in large Petri dishes contained sterile moistened blotting paper A drop of zoospore suspension of the most virulent isolate (PPaL 1) was placed on the upper surface of the leaves after pinprick injury The dishes were kept in a plastic tray containing sterile water and the tray was covered with moistened polythene sheet to provide humid condition for symptom development

Table 1 Plants used for host range studies

Sl.No	Common name	Scientific name
1	Arecanut	*Areca catechu* L
2	Coconut	*Cocos nucifera* L
3	Cocoa	*Theobroma cacao* L
4	Rubber	*Hevea brasiliensis* Mull Arg
5	Black pepper	*Piper nigrum* L
6	Cardamom	*Elettaria cardamomum* Maton
7	Camboge	*Garcinia gummi-gutta* L
8	Vanilla	*Vanilla planifolia* Andr
9	Rose	*Rosa domestica* Mill
10	*Coreopsis*	*Coreopsis lanceolate* L
11	*Eucalyptus*	*Eucalyptus citriodora* L
12	Taro	*Colocasia esculenta* L
13	Acid lime	*Citrus lemon* L

3 9 1.2 Culture disc method

Culture disc of different isolates of the pathogen was inoculated on the leaves of the various hosts as mentioned under 3 3 1

3 9.2 Artificial inoculation under *in vivo* condition

Seedlings of selected host plants, except for vanilla, *Coreopsis* and *Citrus* in which detached leaves were used for inoculation The culture disc of the most virulent isolate (PPaL-1) was inoculated on the leaves as mentioned under 3 3 1 Seedlings were kept in the moistened polythene bags and observations on symptom development were recorded at 24 h intervals

3 10 Cross infectivity of various *Phytophthora* spp. on nutmeg

Various *Phytophthora* spp *viz P palmivora* of coconut and cocoa, *P meadii* of arecanut, rubber, cardamom and vanilla and *P capsici* of black pepper, *P colocasiae* of *Colocasia* and *P citrophthora* of *Citrus*, were inoculated on the leaves of nutmeg seedlings to find out their infectivity on this plant Inoculated plants were kept under humid condition for a period of one month for symptom development

In addition, the infectivity of selected *Phytophthora* spp were also tested on rose, *Eucalyptus, Coreopsis* and *Citrus* which were found to be the hosts of *Phytophthora* isolate of nutmeg

3 11 Identification of *Trichoderma* sp isolated from nutmeg

Trichoderma (TN 1, TN 2 and TN-3) isolated from nutmeg rhizosphere soil were sent to National Centre for Fungal Taxonomy (NCFT), New Delhi for species level identification

3.12 Management of leaf fall disease of nutmeg

Efficacy of the selected fungicides and antagonists were tested against the most virulent *Phytophthora* isolate under *in vitro* and *in vivo* conditions

3.12 1 *In vitro* evaluation of fungicides

Details of the fungicides used for the *in vitro* evaluation are given in the Table 2

Efficacy of eight chemicals were studied by the poisoned food technique (Zentmyer, 1955) Hundred ml of potato dextrose agar medium was taken in 250ml conical flask and sterilized at 1 05 kg/cm^2 pressure for 20 min The chemicals were mixed separately with the medium in suitable proportion to get the desired concentrations and poured to sterilized Petri dishes @ 20 ml/ plate Eight mm sized disc from five day old culture of the pathogen was placed at the center of each Petri dish containing poisoned medium Plates without the fungicide served as control Three replications were maintained for each fungicide Observations were recorded till the control plates attained full growth of the pathogen The per cent inhibition of pathogen was calculated using the formula suggested by Vincent (1927)

$$\text{Per cent inhibition of pathogen} = \frac{C - T}{C} X\ 100$$

C = Growth of the pathogen in control

T = Growth of the pathogen in treatment

3 12.2 *In vitro* evaluation of antagonists against *Phytophthora* isolate of nutmeg

Three *Trichoderma* spp isolated from nutmeg rhizosphere soil and three reference culture *viz T viride* of KAU, *T harzianum* of IISR and *Pseudomonas fluorescens* of KAU were screened for their antagonistic activity against the pathogen *Phytophthora*, by employing dual culture technique (Johnson and Curl, 1972)

Table 2. Fungicides used for *in vitro* evaluation

Chemical name	Trade name	Concentrations	
Copper sulphate + copper hydroxide	Bordeaux Mixture	1%	-
Copper hydroxide 77 WP	Kocide	1 5g/l	2g/l
Copper oxychloride 50 WP	Fytolan	2g/l 2 5g/l	
Cymoxanil 8% + mangozeb 64%WP	Curzate M8	1 5g/l	2g/l
Iprovalicarb 5 5% + propineb 61 3% WP	Melody Duo	1 5g/l	2g/l
Potassium phosphonate 50%	Akomin -50	3ml/l	-
Carbendazim12+mancozeb 63% WP	Saaf	2g/l	-
Potassium phosphanate + phytoalexin	Plant activator	3ml/l	-

3 12.2 1 *In vitro* evaluation of fungal antagonists against pathogen

Antagonistic activity of three *Trichoderma* sp isolated from nutmeg, and two reference cultures *viz T viride* of KAU and *T harzianum* of IISR were tested against the pathogen, adopting deferred antagonism method Sterilised Petri dishes containing PDA medium were inoculated with 8mm mycelial disc of five day old culture of the pathogen at 2 cm from the periphery After 48 h of incubation, 8mm disc of five day old culture of antagonist was placed at the opposite end in the same plate, at 2 cm distance from the periphery Monoculture of the pathogen served as control Three replications were kept for each antagonist and observations were recorded daily till the control plates were fully covered with the fungal growth and per cent inhibition of the pathogen was calculated

The types of interaction between pathogen and the antagonists were recorded using the key developed by Webber and Hedger (1986) consisting of the following types of interactions

1 Intermingling of the hyphae
2 Over growth of the antagonist on the pathogen
3 Mutual inhibition with pigmented band at the point of contact
4 Mutual inhibition with a clear zone between the colonies
5 Extreme inhibition of the pathogen

3.12 2 2 *In vitro* evaluation of bacterial antagonist against pathogen

The bacterial antagonist, *Pseudomonas fluorescens* of KAU was evaluated for its antagonistic activity against *Phytophthora* by simultaneous antagonism method Eight mm mycelial disc of five day old culture of pathogen was inoculated at the center of the PDA mediated Petri dish and the bacterial antagonist was streaked on either side of the pathogen at 2 cm from the periphery of the dish The pathogen grown as monoculture served as control The inoculated plates in triplicates were incubated at room temperature and observation was recorded daily till the full growth of the pathogen in control plates The per cent inhibition of growth of pathogen was calculated by the formula suggested by Vincent (1927)

Based on the per cent inhibition the pathogen, the efficient antagonists were selected for further studies

3.12.3 Management of leaf fall disease of nutmeg under *in vivo* condition

An *in vivo* experiment was conducted during August - September, 2014 to find out the efficacy of fungicides and antagonists, on the management of leaf fall disease Eight fungicides which showed cent per cent inhibition of the pathogen, one efficient *Trichoderma* sp isolated from nutmeg and the efficient standard bioagent selected from the *in vitro* study, were tried using three month old seedlings The experiment details are given below

Design	CRD
No of treatments	22
Replication	3
No of plants / replication / treatment	10
Variety	Local

Two methods of inoculations were adopted based on the method of treatments In treatments which employ spraying, pathogen was inoculated on the lower surface of leaves with culture disc as mentioned in 3 3 1 In soil drenching, inoculum was applied to the collar region of the seedlings as mentioned in 3 5, whereas in spraying + soil drenching treatments, inoculum was applied to foliage and soil Inoculated plants were covered with the moistened polythene bags to provide humidity Antagonists were applied ten days before challenge inoculation of the pathogen and chemical treatments were given on symptom appearance (2 DAI) The details of the treatments are presented in Table 3

3 12.3 1 Observations recorded

Observations on disease incidence, severity and leaf fall were recorded at 10, 15 and 20 days after inoculation

Per cent disease incidence was calculated by the following formula

$$PDI = \frac{\text{Number of plants infected}}{\text{Total number of plants observed}} X\ 100$$

Disease severity was scored using 0-5 scale score chart mentioned below

Grade	Description
0	No infection
1	< 10% leaf area infected
2	>10-25 % leaf area infected
3	>25-50 % leaf area infected
4	>50-75 % leaf area infected
5	> 75 % leaf area infected / Defoliation

Per cent disease severity was calculated using the formula suggested by Wheeler (1969)

$$PDS = \frac{\text{Sum of all numerical ratings}}{\text{Total number of leaves assessed x maximum disease grade}} X100$$

3 12 4 Statistical analysis

Analysis of variance was performed on the data collected in various experiments using the statistical package MSTAT (Freed, 1986) Multiple comparisons among treatment means were done using DMRT

Table 3 Treatments details of the experiment

Treatment No	Treatment details
T_1	Spraying of 1% Bordeaux mixture
T_2	Spraying of copper oxychloride (2 5g/l)
T_3	Spraying of copper hydroxide (2g/l)
T_4	Spraying of cymoxanil 8% + mancozeb 64% WP (2g/l)
T_5	Spraying of iprovalicarb 5 5% +propineb 61 3% WP (1 5g/l)
T_6	Spraying of potassium phosphonate 50% (3ml/l)
T_7	Spraying of carbendazim 12 %+mancozeb 63% WP (2g/l)
T_8	Spraying of plant activator (3ml/l)
T_9	Soil drenching of copper oxychloride (2g/l)
T_{10}	Soil drenching of copper hydroxide (2g/l)
T_{11}	Soil drenching of cymoxanil 8% + mancozeb 64% WP (2g/l)
T_{12}	Soil drenching of iprovalicarb 5 5% +propineb 61 3% WP (2g/l)
T_{13}	Spraying of 1% Bordeaux mixture + soil drenching of copper oxychloride (2 5g/l)
T_{14}	Spraying of 1% Bordeaux mixture + soil drenching of copper hydroxide (2g/l)
T_{15}	Spraying and soil drenching of copper oxychloride (2 5g/l)
T_{16}	Spraying and soil drenching of copper hydroxide (2g/l)
T_{17}	Spraying of 1% Bordeaux mixture and soil application of *T viride* (KAU)
T_{18}	Prophylactic spray of 2% *P fluorescens* and soil application of *T viride* (KAU)
T_{19}	Soil application of *T viride* 1 of nutmeg
T_{20}	Soil application of *T viride* (KAU)
T_{21}	Control (inoculation of pathogen on leaves)
T_{22}	Control (soil application of pathogen)

Results

4. RESULTS

The experimental results obtained from the studies on "Phenotypic and molecular characterisation of *Phytophthora* sp inciting leaf fall of nutmeg" are presented below

4 1 Collection of samples

Eighteen diseased samples were collected from seventeen locations, of which, sixteen were leaves, collected from Parakkadavu, Kodissery, Poovathussery, Mambra, Venoor and Koottala of Thrissur district, Kalady, Mattur, Thevarmadom, Sreemoolanagaram, Thuravoor, Kanjoor, Desam and Koothattukulam of Ernakulam district, Vaikom and Kuravilangad of Kottayam district and two were fruit samples from Parakkadvu and Mookkanoor of Thrissur district (Table 4) (Plate 1)

4 2 Isolation of the pathogen

White fungal growth was observed around the infected tissues on carrot agar medium and the pathogen associated with the disease was found to be *Phytophthora* based on cultural and morphological characters (Plate 2)

The isolates were named representing the name of pathogen, place of collection, plant part used for isolation and the Arabic numerals in serial order The isolate PPaL-1 indicates - 'P' stands for *Phytophthora* Pa represents the place, Parakkadavu, 'L' denotes the leaf and '1' is the Arabic numeral (Table 4)

4 3 Pathogenicity

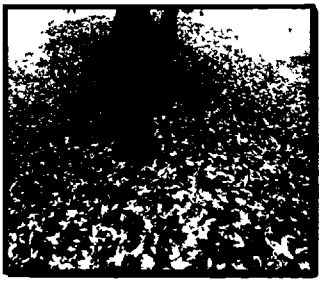
The preliminary study on pathogenicity of Mambra isolate of the pathogen on detached twigs under lab condition showed typical symptoms on leaves, petiole, shoot and fruits as observed in natural condition (Plate 3)

Leaves and petiole showed infection at two days after inoculation on artificial inoculation with culture disc Typical dark brown water soaked lesions appeared on the moculated midrib area of the leaves which later enlarged and

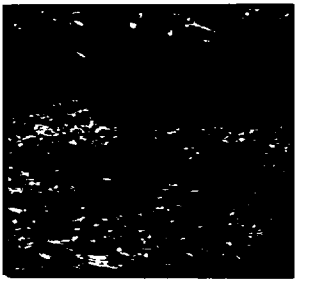
Table 4. Locations of the sample collection

Sl No	Location	Isolates No
1	Parakkadavu	PPaL-1
2	Kodissery	PKoL-2
3	Poovathussery	PPoL-3
4	Mambra	PMaL-4
5	Venoor	PVeL-5
6	Koottala	PKtL-6
7	Kalady	PKaL-7
8	Mattur	PMtL-8
9	Thevarmadom	PThL-9
10	Sreemoolanagaram	PSrL-10
11	Thuravoor	PTuL-11
12	Kanjoor	PKnL-12
13	Desam	PDeL-13
14	Koothattukulam	PKkL-14
15	Vaikom	PVaL-15
16	Kuravilangad	PKuL-16
17	Parakkadvu (fruit)	PPaF 17
18	Mookkanoor (fruit)	PMoF-18

Plate 1. Collection of samples

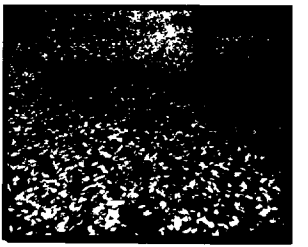


Kalady

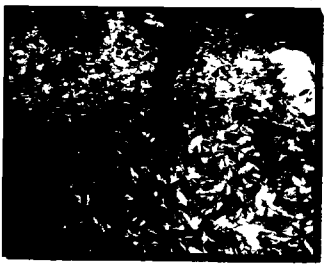


Mattur

Thevarmadom



Kanjoor



Poovathussery

Plate 2. Isolation of the pathogen

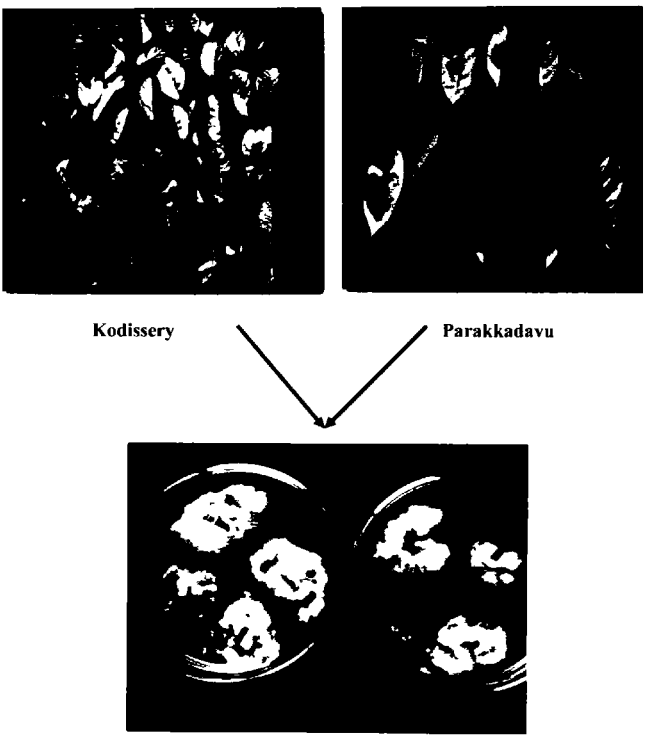


Plate 3. Pathogenicity - Under lab condition

On leaf

On petiole

On shoot

On fruit

spread to the petiole On petiole, infection started as brown lesion and then spread to the entire leaf lamina Infection was also noticed on the twig and at the petiole junction Defoliation observed at 3 DAI On young shoots, the symptom appeared as dark brown discolouration resulting in rotting and drying of the entire stem Symptoms also appeared on fruits at 3 DAI as dark brown water soaked lesions, which enlarged and caused rotting and splitting of fruits

On zoospore inoculation, symptoms appeared at 3 4 DAI, as dark brown water soaked lesion all over the leaf lamina Leaf petiole and shoots showed dark brown discolouration and leaves defoliated in 10 12 DAI On fruits, dark brown lesions appeared at stalk regions which spread all over the rind leading to rotting and splitting of fruits Infection spread to inner pericarp, kernel and mace resulted in rotting of these parts Reisolation of the fungus from various infected part and confirmed the identity of the pathogen culturally and morphologically and thus fulfilled Koch postulates

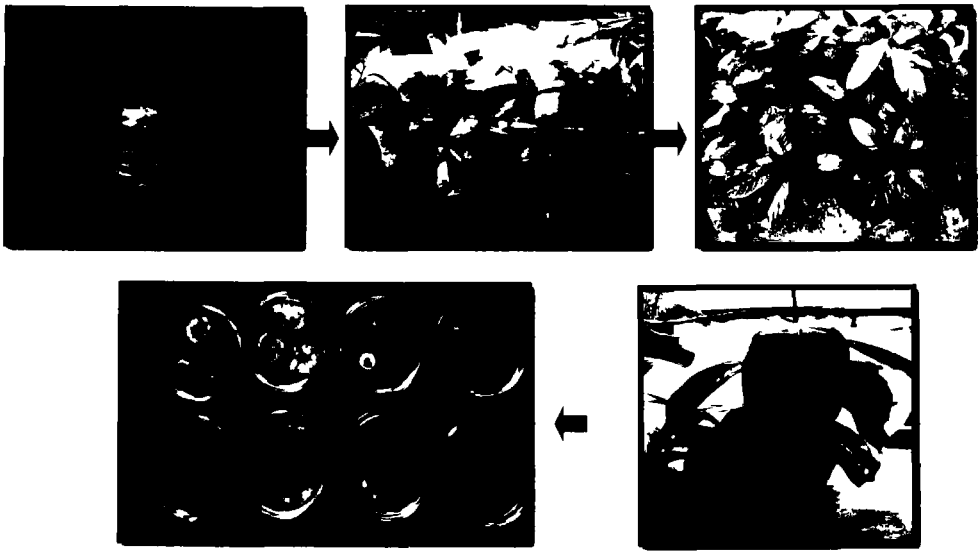
4 3.1 Pathogenicity of different isolates of the pathogen

Inoculation of 18 isolates of the pathogen with culture disc and zoospore suspension also showed typical symptoms on leaves and shoot of nutmeg seedlings and on detached fruits, as noticed in natural condition Reisolation from the infected parts yielded the pathogen having similar characters as that of original cultures and thus proved the pathogenicity of different isolates also (Plate 4, 5& 6)

Infection was observed with both inoculation methods, as well as on both leaf surfaces and with/without injury However, days for symptom expression varied with all these factors Early infection was noticed with culture disc as compared to zoospore inoculation Inoculation of culture disc on lower leaf surface with injury, showed, symptom expression in 1 2 DAI, where it was 4-6 days in without injury Inoculation with culture disc on upper surface with injury took infection within 1- 4 days while it was 5-7 days with non injury However, zoospore inoculation on lower and upper leaf surface with injury showed symptom appearance only at 7-8 and 10 DAI respectively

Plate 4. Pathogenicity of *Phytophthora* isolates under *in vivo* condition

Culture disc method - On lower surface

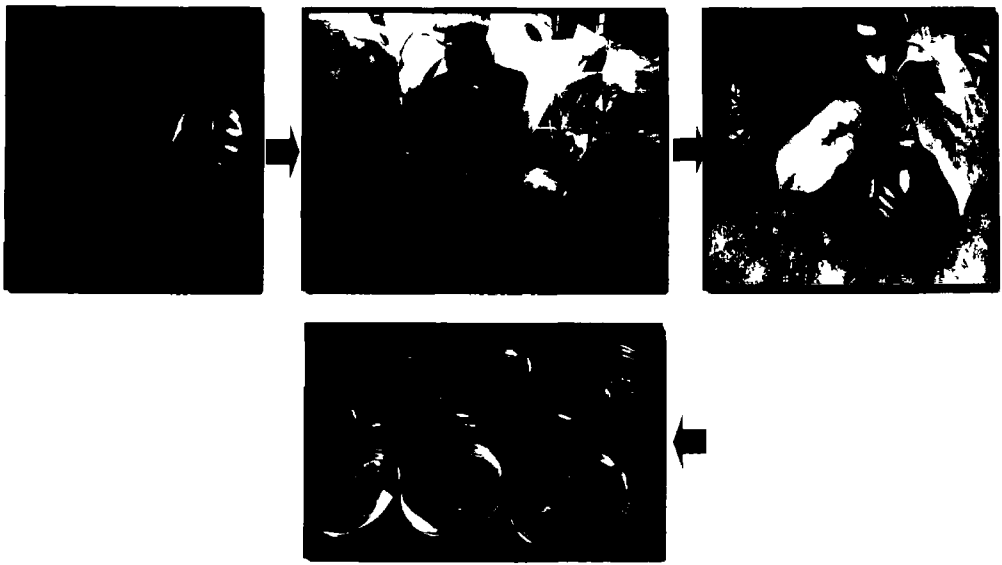


Reisolation of isolates of pathogen

Symptom on nutmeg seedlings

Plates 5. Pathogenicity of *Phytophthora* isolates under *in vivo* condition

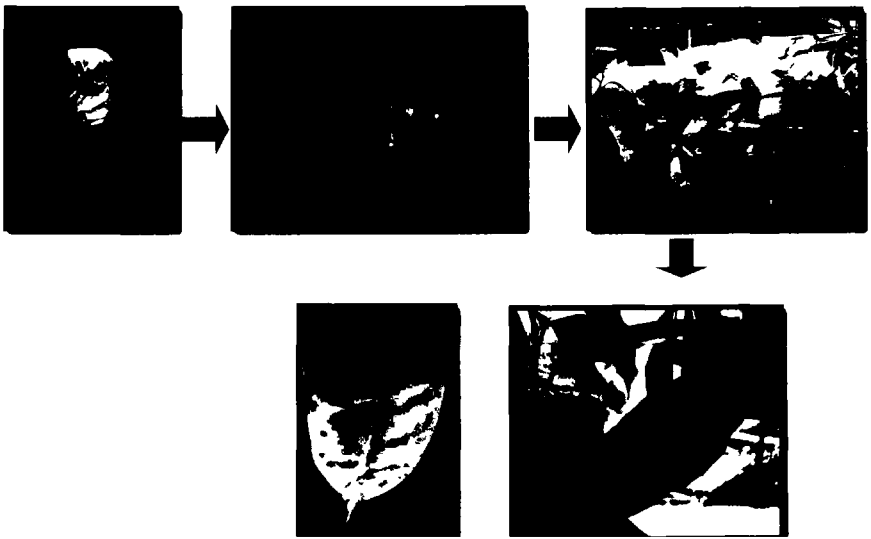
Culture disc method - On upper surface



Reisolation of isolates of pathogen

Plates 6. Pathogenicity of *Phytophthora* isolates under *in vivo* condition

Zoospore inoculation



From the above results, it is observed that, early infection was obtained with inoculation of culture disc on lower leaf surface with pin prick injury

4 4 Symptomatology

Symptomatology of the disease on various parts of nutmeg was studied under both natural and artificial conditions

4 4 1 Symptoms under natural conditions

General symptom observed was the severe premature defoliation during the South - West monsoon period (Plate 7A) Infection was observed on leaves, shoots and fruits, but no infection was noticed on roots

4.4 1 1 Symptoms on leaves

Symptom first appeared as dark brown water soaked lesion mainly on the midrib of the leaves which later enlarged and spread along the lateral veins to leaf lamina resulted in blighting and premature defoliation (Plate 7B 1&2) In some cases, dark brown lesions were noticed at the tip/ the margins of the leaves also (Plate 7B 3&4) Lesion spread very rapidly under high humid condition Petioles of the infected leaves showed black discoloration (Plate 7B 5&6)

4 4.1 2 Symptoms on shoot

Black water soaked lesions were observed on young shoots which enlarged in size resulting in rotting and drying up of shoots from the tip to downwards and resulted in die back of young shoots (Plate 8)

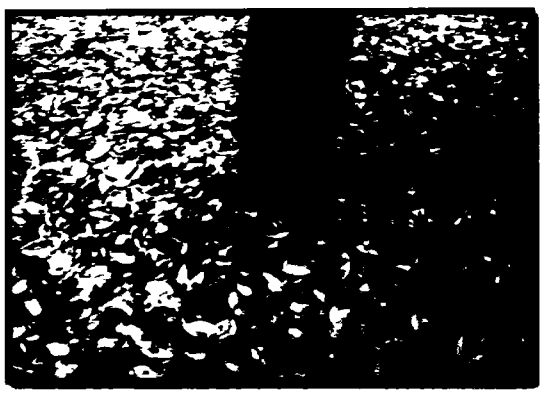
4 4 1.3 Symptoms on fruits

The symptoms on fruits appeared as water soaked lesions on fruit surface, which later spread all over the area resulting in rotting of the rind which got separated from the internal tissues As the infection progressed, rotting spread to pericarp, mace and kernel and emitted foul smell Infected fruits showed cottony fluffy mycelial growth on outside and inside the fruits Black discolouration was also noted on fruit stalk Affected fruits splitted and dropped off prematurely (Plate 9)

Plates 7. Symptomatology

Under natural condition

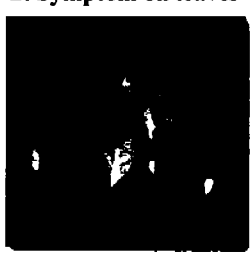
A. General symptom



Heavy defoliation

B. Symptom on leaves

1. Midrib



2. Different stages of infection

3. Leaf tip

4. Leaf margin

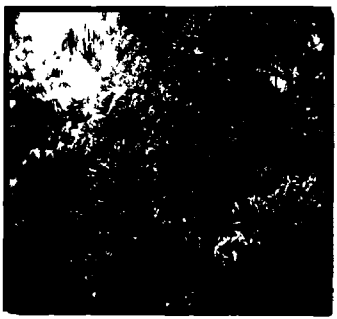
5. Petiole infection

6. Defoliated leaves

Plate 8. Symptomatology

Symptoms on shoot

Shoot rot



Dieback

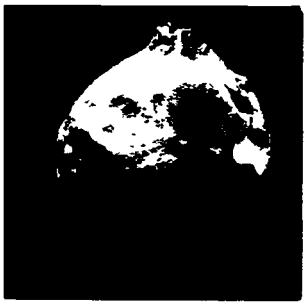
Defoliated tree

Plate 9. Symptomatology

Symptoms on fruits

Fruit fall

Fungal growth incide the fruit



Fungal growth outside the fruit

4 4 2 Symptoms under artificial condition

Symptomatology of different *Phytophthora* isolates on nutmeg seedlings and detached fruits were studied under artificial condition during the month of July Artificial inoculation was done separately on leaves, shoots, roots and fruits and symptoms on each part were recorded All 18 isolates showed same type of symptoms on various plant parts as observed under natural condition

4.4 1.4 Symptoms on leaves

Initial symptom developed within 1 2 days after inoculation as dark brown to black water soaked lesion on the midrib region followed by yellowing of vein and veinlets of inoculated leaves Later, lesions enlarged and spread along the lateral veins to leaf lamina resulted in blighting/rotting Yellow halo was noticed around the blighted area and petioles of the infected leaves showed black discolouration (Plate 10)

Days for initial infection, intensity of yellow halo and days to leaf fall varied with different isolates and detailed in Table 5 Initial symptom was developed one day after inoculation in all isolates except isolate PKoL-2 & PKtL 6 which took two days Yellow halo was observed around the lesion, but intensity varied among the isolates Deep yellow with highly prominent halo was observed in PKoL-2, PVeL 5, PKnL-12 and PVaL-15 and yellow with prominent halo was noticed in PMtL-8, PThL-9, PSrL 10, PTuL-11, PKkL-14 and PMoF 18 But the isolates, PPaL-1, PPoL 3, PMaL 4, PKtL-6, PKaL 7, PDeL 13, PKuL-16 and PPaF-17 showed pale yellow with less prominent halo and the defoliation of green leaves occurred within 3-5 days of inoculation, which varied with the isolates

Plate 10. Symptomatology

Under artificial condition

Symptoms of various *Phytophthora* isolates on leaves

PPaL-1

PKoL-2

PPoL-3

PMaL-4

PVeL-5

PKtL-6

PKaL-7

PMtL-8

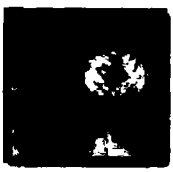
PThL-9

PSrL-10

PTuL-11

PKnL-12

PDeL-13



PKtL-14

PVaL-15

PKuL-16

PPaF-17

PMoF-18

Table 5 Variation in symptom development on nutmeg by different isolates of the pathogen

Isolate No	On leaves			On shoots		On fruits	
	Days for initial infection	*Lesion halo	Days to leaf fall	Days for initial infection	Days for die back	Days for initial infection	Days for complete rotting
PPaL-1	1	+	3	4	14	5	12
PKoL-2	2	+++	5	5	16	6	14
PPoL-3	1	+	4	5	14	5	12
PMaL-4	1	+	3	4	14	5	12
PVeL-5	1	+++	3	4	14	5	12
PKtL-6	2	+	4	4	14	5	12
PKaL-7	1		4	5	16	6	14
PMtL-8	1		4	4	14	5	12
PThL-9	1		4	4	14	6	14
PSrL-10	1		3	4	14	7	15
PTuL 11	1		3	4	14	6	14
PKnL-12	1		3	4	14	5	12
PDeL-13	1		4	4	14	6	12
PKkL-14	1		5	5	16	7	14
PVaL-15	1		4	4	14	7	14
PKuL-16	1		4	4	10	6	14
PPaF-17	1		3	4	14	4	10
PMoF-18	1		4	5	16	5	12

*** Lesion halo**

+++ - Deep yellow and highly prominent

++ - Yellow and prominent

+ - Pale yellow and less prominent

4 4.1 5 Symptoms on shoots

Initial infection was observed in 4 5 days after inoculation as dark brown discoloration on the inoculated area, which later spread upward and downward of the shoots, then to petiole and finally to leaves through midrib As the infection advanced, tender shoots showed rotting and led to drying up of shoots which occurred within 10 16 DAI Days for initial infection and die back of shoots varied with isolates (Table 5) Early dieback symptom was noticed in PKuL 16 isolate (10 DAI) (Plate 11)

4.4 1.6 Symptoms on fruits

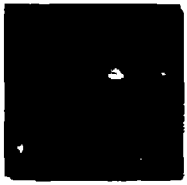
Initial symptoms were noticed after 4- 7 days of inoculation depending up on the isolates Symptom first initiated as light brown discolouration near the stalk end of the inoculated fruits, which later turned to dark brown water soaked lesions and fruit splitting was observed at this stage Water soaked lesions enlarged rapidly resulted in rotting of rind, then spread to pericarp, mace and kernal White mycelial growth was found on all infected parts of the fruits Rotted fruits emitted foul smell (Plate 12) Days for initial infection and for complete rotting of fruit varied among the isolates (Table 5) Early infection (4 DAI) and complete rotting (10 DAI) was observed in PPaF-17, which is an isolate from the fruit and the isolate PSrL 10 showed maximum days for infection and complete rotting of the fruit

4 4 1 7 Symptoms on root inoculation

Drying up of plant parts was observed on seedlings by inoculation on root Drying up started from tip of the leaves at 6 DAI Later, prominent yellow halo was developed around the necrotic area and drying up spread to all other leaves Dried leaves remained attached to the plant and inoculated plants completely dried up after 30 days of inoculation (Plate 13A)

Plate 11. Symptomatology

Symptoms of various *Phytophthora* isolates on shoot



PPaL-1

PKoL-2

PPoL-3

PMaL-4

PVeL-5

PKtL-6

PKaL-7

PMtL-8

PThL-9

PSrL-10

PTuL-11

PKnL-12

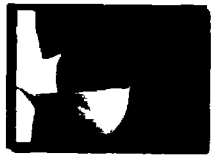
PDeL-13

PKtL-14

PVaL-15

PKuL-16

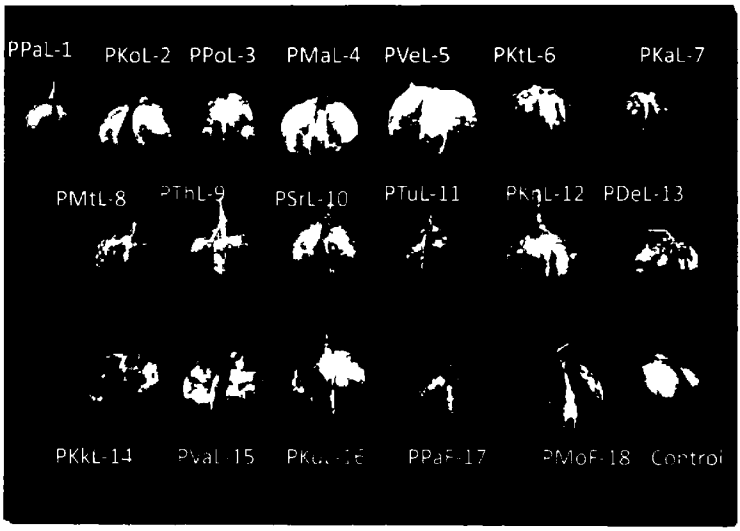
PPaF-17



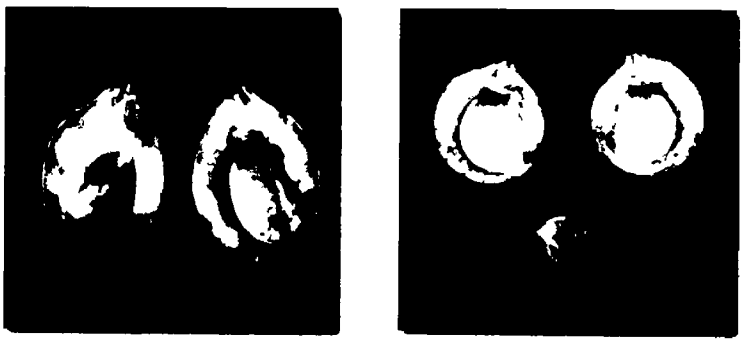
PMoF-18

Plate 12. Symptomatology

Symptoms of various *Phytophthora* isolates on fruit



Infection on fruit



Fungal growth on pericarp, kernel and mace

4 5 Study on virulence of various *Phytophthora* isolates of nutmeg

Result of the preliminary study conducted on the virulence of 18 isolates on detached leaves is given in Table 6 Observation on lesion area produced by various isolates revealed that, there is variability in virulence among the isolates (Plate 13B) The early infection (1 DAI) was noticed in all isolates except PKtL 6, PMtL-8 and PKkL-14 which showed infection on 2 DAI and in PKoL 2 on the third day only It is also found that, PPaL-1 recorded maximum lesion area of 4 5 cm^2 which was followed by PPaF-17 on third day after inoculation which are the isolates from Parakkadavu The minimum lesion area was noticed in PKoL-2 On 7th day after inoculation, maximum lesion spread was observed in PPaL-1 followed by PPaF-17 and PSrL-10, and was less in PKoL-2, indicating PPaL-1 and PPaF-17 as highly virulent and PKoL-2 as least virulent

Variation in virulence was further confirmed by artificial inoculation with 18 isolates on both lower and upper leaf surfaces of nutmeg seedlings Observations on lesion area, per cent leaf fall and days to leaf fall recorded at different intervals revealed the variability in virulence among the isolates (Table 7 to 10)

In case of artificial inoculation on lower leaf surface, early infection (1 DAI) was observed in all isolates except PKoL-2 and PKtL 6 which have taken 2 days for infection (Table 7) At 3 DAI, isolate PPaL-1 recorded lesion size of 4 8 cm^2 and followed by PPaF-17 with 4 6 cm^2 whereas minimum lesion area (2 1 cm^2) was noticed with PKoL-2 and PKtL-6 It is also noted that, there is variation in days to leaf fall among the isolates Early leaf fall (3 DAI) was noticed in PPaL-1, PMaL-4, PVeL-5, PSrL-10, PTuL-11, PKnL-12 and PPaF 17 whereas, PKoL 2 and PKkL-14 showed leaf fall only at 5th day and all others recorded leaf fall symptom at 4 DAI

Data on per cent leaf fall furnished in Table 8 showed that, of the seven isolates which showed early leaf fall at 3DAI, maximum leaf fall (60 per cent) was noticed in PPaL 1 and the minimum (33 33 per cent) in PVeL 5 and PKnL 12

Table 6 Differential response of *Phytophthora* isolates of nutmeg with artificial inoculation on detached leaves

Isolates	Lesion area (cm^2)						
	Days after inoculation						
	1	2	3	4	5	6	7
PPaL-1	0 6	2 0	4 5	6 6	8 7	10 5	12 5
PKoL-2	-	-	0 6	1 7	3 1	5 8	7 6
PPoL-3	0 5	1 8	4 2	6 3	8 2	10 3	12 1
PMaL-4	0 4	1 8	4 1	6 2	7 7	10 5	12 2
PVcL-5	0 6	2 0	4 1	6 0	8 0	10 1	12 0
PKtL-6		0 4	2 3	4 4	6 6	8 9	10 7
PKaL-7	0 4	1 5	4 2	6 2	8 4	10 2	12 0
PMtL-8	-	0 3	3 1	4 5	7 5	9 3	11 5
PThL-9	0 4	1 3	3 9	5 8	8 0	9 6	11 4
PSrL-10	0 4	1 5	4 0	6 0	8 1	10 3	12 3
PTuL-11	0 5	1 7	3 6	5 4	7 5	9 4	10 8
PKnL-12	0 5	1 7	3 6	5 7	7 8	9 4	10 6
PDeL-13	0 4	1 6	3 4	5 2	7 1	9 0	11 1
PKkL-14		0 4	3 5	4 9	6 4	8 5	10 2
PVaL 15	0 6	1 7	2 5	4 3	7 1	9 2	11 3
PKuL-16	0 3	1 5	2 4	4 3	7 3	9 4	10 6
PPaF-17	0 5	2 0	4 3	6 2	8 5	10 3	12 3
PMoF 18	0 3	1 6	3 4	5 6	7 6	9 4	11 2

Plate 13. Symptomatology

A. Symptoms on root inoculation

B. Studies on virulence of *Phytophthora* isolates on detached leaves

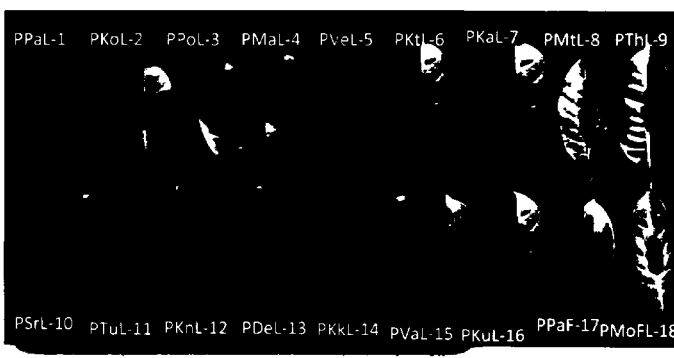


Table 7. Differential response of *Phytophthora* isolates with artificial inoculation on lower leaf surface of nutmeg seedlings

Isolates	Lesion area (cm^2) Days after inoculation					Days to leaf fall
	1	2	3	4	5	
PPaL 1	0 7	2 2	4 8	-	-	3
PKoL-2	-	0 7	2 1	4 6	6 1	5
PPoL-3	0 8	2 0	4 3	6 1	-	4
PMaL-4	0 5	2 1	4 5	-	-	3
PVeL-5	0 8	2 1	4 5	-	-	3
PKtL-6	-	0 5	2 1	4 3	-	4
PKaL 7	0 5	2 0	4 1	5 1	-	4
PMtL-8	0 4	1 0	3 2	5 1		4
PThL 9	0 5	1 7	3 9	6 2		4
PSrL 10	0 5	1 6	3 8	-	-	3
PTuL 11	0 6	1 7	3 9			3
PKnL-12	0 6	2 1	3 7	-		3
PDeL-13	0 5	1 7	4 1	6 7		4
PKkL-14	0 4	1 4	3 9	5 7	6 8	5
PVaL-15	0 7	1 8	4 0	6 2	-	4
PKuL-16	0 4	1 0	3 2	5 1	-	4
PPaF-17	0 6	2 1	4 6	-		3
PMoF-18	0 6	1 5	3 9	6 1		4

Table 8. Per cent leaf fall by artificial inoculation of *Phytophthora* isolates on lower leaf surface

Isolates	Per cent leaf fall				
	Days after inoculation				
	3	4	5	6	7
PPaL-1	60 00	80 00	86 66	98 33	100 00
PKoL-2	-		53 33	86 66	98 33
PPoL-3	-	46 66	73 33	93 00	98 33
PMaL-4	46 66	60 00	86 66	93 00	98 33
PVeL 5	33 33	46 66	73 33	86 66	98 33
PKtL 6	-	53 33	80 00	90 00	96 66
PKaL-7	-	53 33	73 33	93 00	98 33
PMtL 8	-	60 00	73 33	80 00	93 00
PThL 9	-	60 00	80 00	96 33	98 33
PSrL-10	53 33	80 00	86 66	96 33	98 33
PTuL-11	40 00	66 66	80 00	93 00	96 33
PKnL 12	33 33	53 33	73 33	86 66	96 00
PDeL-13	-	46 66	73 33	82 66	96 33
PKkL-14		-	46 66	73 33	80 00
PVaL-15		73 33	86 66	96 33	98 33
PKuL-16	-	53 33	80 00	93 00	98 33
PPaF-17	53 33	73 33	86 66	93 00	98 33
PMoF 18	-	33 33	53 33	73 33	80 00

At 5th day after inoculation, all isolates showed leaf fall symptom in which maximum per cent leaf fall of 86 66 was observed in PPaL 1, PMaL 4, PSrL 10, PVaL 15 and PPaF-17 and minimum (53 33 per cent) in PMoF-18 Cent per cent leaf fall was recorded in PPaL 1 on the seventh day of inoculation

Data on inoculation on upper leaf surface (Table 9) revealed that, early infection was observed only in six isolates *viz* PPaL 1, PThL-9, PSrL 10, PTuL 11, PKnL-12 and PPaF-17 The infection was noticed in all the isolates on 2 DAI except PKoL-2 and PKtL 6, which recorded infection only on 4 and 3 DAI respectively At 4 DAI, the maximum lesion area of 5 9 cm^2 was recorded for PPaL-1 and the minimum (2 1 cm^2) with PKtL-6 It is also noted that, leaf fall was observed in the isolates PPaL 1, PThL-9, PSrL 10, PTuL-11, PKnL-12 and PTaF-17 on the 4th day of inoculation, whereas, it was on the 6th day in PKoL-2 and all others exhibited leaf fall on the fifth day of inoculation

Data on per cent leaf fall furnished in Table 10 showed variation among the isolates Leaf fall was noticed only at 4th day of inoculation with six isolates, of which, PPaL-1 recorded maximum per cent of 46 66 and minimum (20 per cent) with PKnl-12 From 5th day onwards all isolates showed leaf fall except PKoL 2 On 7 DAI, the maximum per cent leaf fall (96 33) was recorded with the isolate PPaL-1 while the lowest (80 per cent) was in PKoL 2 and PMoF 18

Comparing the response of 18 *Phytophthora* isolates on nutmeg leaves with inoculation on both leaf surfaces, variation among the isolates was noticed with respect to early infection and leaf fall It was observed that of the 18 isolates, PPaL-1, PThL 9, PSrL-10, PTuL-11, PKnL-12 and PPaF-17 showed infection in 24 h with inoculation on lower and upper leaf surface and in both cases, late infection was noticed m PPoL 2 which took 2 and 4 days respectively There were variations in days to leaf fall, which was 3-5 and 4 6 DAI for lower and upper leaf surfaces respectively with maximum per cent leaf fall in PPaL 1 and minimum in PMoF 18 at 7 DAI

Table 9 Differential response of *Phytophthora* isolates with inoculation on upper leaf surface

Isolates	Lesion area (cm^2)						Days to leaf fall
	Days after inoculation						
	1	2	3	4	5	6	
PPaL 1	0 4	2 0	4 2	5 9	-	-	4
PKoL-2	-	-	-	0 9	1 8	3 7	6
PPoL-3	-	0 9	2 5	4 9	6 7	-	5
PMaL-4		0 8	2 1	4 6	6 9		5
PVeL-5		0 6	1 9	3 1	5 2	-	5
PKtL-6	-	-	0 8	2 1	4 6		5
PKaL-7		0 7	2 1	3 8	5 5		5
PMtL-8		0 5	1 8	3 2	5 1		5
PThL-9	0 3	1 9	4 1	5 8	-	-	4
PSrL-10	0 3	1 9	4 2	5 8	-	-	4
PTuL-11	0 5	1 5	3 6	5 6	-	-	4
PKnL-12	0 6	1 7	3 3	5 7			4
PDcL-13	-	0 8	2 5	4 2	6 1	-	5
PKkL-14	-	1 0	2 3	4 1	5 8	-	5
PVaL-15	-	0 5	1 5	3 4	5 1	-	5
PKuL 16	-	0 7	2 3	4 2	6 3		5
PPaF 17	0 5	1 7	3 4	4 7	-		4
PMoF-18		0 6	1 8	3 2	5 3	-	5

Table 10 Per cent leaf fall by inoculation of *Phytophthora* isolates on upper leaf surface

Isolates	Per cent leaf fall			
	Days after inoculation			
	4	5	6	7
PPaL-1	46 66	73 33	86 66	96 33
PKoL 2	-	-	53 33	80 00
PPoL-3		60 00	80 00	93 00
PMaL-4	-	60 00	82 00	93 00
PVeL-5	-	40 00	60 00	82 00
PKtL 6	-	40 00	73 33	86 66
PKaL-7	-	46 66	73 33	82 00
PMtL-8	-	46 66	73 33	82 00
PThL-9	33 33	53 33	80 00	86 66
PSrL-10	40 00	53 33	80 00	93 00
PTuL-11	33 33	46 66	73 33	86 66
PKnL 12	20 00	46 66	73 33	82 00
PDeL-13	-	46 66	73 33	82 00
PKkL-14	-	53 33	80 00	93 00
PVaL-15	-	40 00	73 33	82 00
PKuL-16	-	60 00	82 00	93 00
PPaF 17	40 00	73 33	86 66	93 33
PMoF 18		46 66	60 00	80 00

The discrete statistical analysis shown in Table 11, indicated that, the isolates PPaL 1, PPaF-17 (Parakkadavu) and PSrL-10 (Sreemoolanagaram) were categorized in Group-3 which is considered as highly virulent based on leaf area infected and per cent leaf fall parameters, while considering the days to leaf fall, highly virulent was categorized as Group-1 in which PPaL-1, PPaF-17 and PSrL 10 were included PKoL 2, the isolate from Kodissery (Thrissur) was least virulent and the rest of the isolates were moderately virulent

Thus this study revealed that, there is variation in virulence among the isolates collected from different locations Based on the maximum lesion area, per cent leaf fall and early leaf fall, which are the criterion considered for the highly virulence in the present study, the isolate PPaL 1 was found to be more virulent under both lab and *in vivo* conditions and hence selected for host range and disease management studies

4.6 Cultural and morphological characteristics of the pathogen

Different isolates were subjected to cultural and morphological studies by using five different media *viz* carrot agar, potato dextrose agar, oat meal agar, coconut water agar and V8 juice agar

4.6.1 Cultural characters

The cultural characters of 18 *Phytophthora* isolates of nutmeg were studied on five different media and are summarized in Table 12 Colony characters of different isolates varied among the five media and both replications showed similar type of growth pattern in all five media tested

4 6 1 1 Carrot agar medium

Variation in colony characters were observed within 18 *Phytophthora* isolates These isolates showed six distinct colony patterns on the carrot agar medium as follows (Veena, 1996, Bhai and Sarma, 2005, Widmer, 2010, Tsopmbeng, 2012)

Table 11 Grouping of *Phytophthora* isolates based on virulence

Inoculation method	Parameters	*Group 1	**Group 2	***Group 3
On lower surface of detached leaves	Lesion area	PKoL-2	PPoL-3, PMaL- 4, PVeL- 5, PKtL- 6, PKaL- 7, PMtL- 8, PThL- 9, PSrL- 10, PTuL- 11, PKnL- 12, PDeL- 13, PKkL- 14, PVaL- 15 PKuL- 16,PMoF- 18,	PPaL-1, PPaF-17
On lower surface of seedling leaves	Lesion area	PKoL-2, PKtL-6	PPoL-3,PMaL- 4,PVeL- 5,PKaL- 7,PMtL- 8, PThL- 9, PSrL- 10, PTuL- 11, PKnL- 12, PDeL- 13, PKkL- 14, PVaL- 15, PKuL- 16 PMoF- 18	PPaL-1, PPaF- 17
	Per cent leaf fall	PKoL-2,PKkL- 14,PMoF- 18	PPoL-3,PVeL- 5,PKtL- 6,PKaL- 7,PMtL- 8,PThL- 9,PTuL- 11,PKnL- 12, PDeL- 13,PKuL-16	PPaL-1 PMaL- 4,PSrL- 10, PVaL- 15,PPaF- 17
On upper leaf surface of seedling leaves	Lesion area	PKoL-2,PKtL-6	PPoL-3,PMaL- 4,PVeL- 5,PKaL- 7,PMtL- 8,PTuL- 11,PKnL- 12 PDeL- 13 PKtL- 14 P15 PKuL- 16,PPaF- 17,PMoF- 18	PPaL-1,PThL- 9,PSrL-10
	Per cent leaf fall	PKoL-2	PPoL-3,PMaL- 4,PVeL- 5,PKtL- 6,PKaL- 7,PMtL- 8,PThL- 9 PSrL- 10, PTuL- 11,PKnL- 12,PDeL- 13 PKkL- 14, PVaL- 15, PKuL- 16, PMoF- 18,	PPaL-1,PPaF-17
	Category	**Least virulent**	**Moderately virulent**	**Highly virulent**
On lower surface of seedling leaves	Days to leaf fall	PPaL-1,PMaL- 4,PVeL- 5, PSrL- 10,PTuL- 11, PKnL- 12,PPaF-17	PPoL-3,PKtL- 6,PKaL- 7,PMtL- 8,PThL- 9,PDeL- 13,PVaL- 15,PKuL- 16,PMoF- 18	PKoL-2 PKkL-14
On upper leaf surface of seedling leaves		PPaL-1,PThL- 9 PSrL- 10, PTuL- 11 PKnL- 12 PPaF-17	PPoL-3,PMaL- 4,PVeL- 5,PKtL- 6,PKaL- 7,PMtL- 8,PDeL- 13, PKkL- 14 PVaL- 15,PKuL- 16,PMoF- 18	PKoL-2
	Category	**Highly virulent**	**Moderately virulent**	**Least virulent**

* ≤ Mean – Standard deviation ** ≤ Mean – Standard deviation to ≥ Mean + Standard deviation *** ≥ Mean + Standard deviation

Table 12 Cultural characters of various isolates of the pathogen on different media

Isolate	Carrot agar	Potato dextrose agar	Oat meal Agar	Coconut water agar	V8 juice agar
PPaL-1	White mycelium with weak rosette pattern	Dense cottony white mycelium with pronounced concentric rings like growth pattern	Sparse dull white mycelium without uniform surface and intermittent lobbed pattern	White sparse aerial mycelium stellate at the center and radiating from center to periphery	Appressed white aerial mycelium with weak rosette pattern
PKoL-2	Cottony white mycelium with distinct rosette pattern				
PPoL 3	White slightly cottony mycelium with intermittent lobbed pattern				
PMaL 4	Cottony white mycelium with distinct rosette pattern				
PVeL-5	Cottony white mycelium with distinct rosette pattern				
PKtL-6	Dull white mycelium without distinct colony pattern				
PKaL-7	Dull white mycelium without distinct colony pattern				
PMtL 8	Dense cottony white mycelium without distinct colony pattern				
PThL 9	Dull white mycelium without distinct colony pattern				
PSrL-10	Cottony white mycelium with distinct rosette pattern				
PTuL-11	White mycelium with weak rosette pattern				
PKnL-12	Dull white mycelium without distinct colony pattern				
PDeL 13	Cottony white mycelium with distinct rosette pattern				
PKkL-14	Cottony white mycelium with distinct rosette pattern				
PVaL-15	White powdery mycelial growth with concentric rings towards the periphery				
PKuL-16	White slightly cottony mycelium with intermittent lobbed pattern				
PPaF-17	White powdery mycelial growth with concentric rings towards the periphery				
PMoF 18	White slightly cottony aerial mycelium with intermittent lobbed mycelial pattern				

Type 1 White mycelium with weak rosette growth pattern, aerial mycelium more or less sparse without defined leading edges (PPaL-1, PTuL 11) – Plate 14a

Type 2 Dull white mycelium without distinct colony pattern, submerged and very sparse aerial mycelium (PKtL-6, PKaL 7, PThL 9, PKnL-12,) – Plate 14b

Type 3 White slightly cottony aerial mycelium with intermittent lobbed (clustered) mycelial pattern without defined leading edges (PKoL-2, PPoL 3, PKuL 16, PMoF-18) – Plate 14c

Type 4 Cottony white aerial mycelium with distinct rosette pattern (PMaL-4, PVeL-5, PSrL-10, PDeL 13, PKkL-14) – Plate 14d

Type 5 No distinct colony pattern, aerial mycelium was profuse giving dense cottony white appearance (PMtL 8) – Plate 14e

Type 6 White powdery mycelial growth with concentric rings towards the periphery (PVaL 15, PPaF-17) – Plate 14f

4.6.1 2 Potato dextrose agar

Colony characters of all 18 isolates showed more or less similar pattern of growth by dense cottony white aerial mycelium with sharply defined leading edges It showed pronounced concentric rings like growth pattern (Plate 15A)

4.6.1 3 Oat meal agar

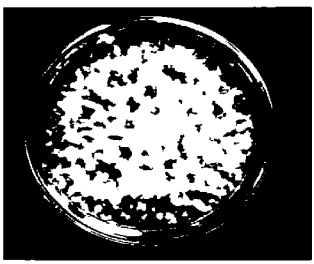
All 18 *Phytophthora* isolates produced sparse dull white aerial mycelial growth without uniform surface Aerial mycelium was more or less absent in the peripheral areas of the colony It also showed small intermittent lobbed mycelial pattern (Plate 15B)

Plate 14. Cultural characters

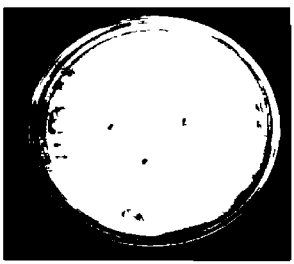
Carrot agar medium

a Type 1 (PPaL-1, PTuL-11)

b Type 2 (PKoL-2, PKtL-6, PMtL-8, PDeL-13)

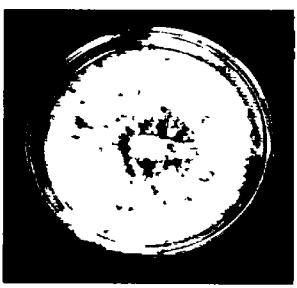


c Type 3 (PPoL-3,PThL-9, PKuL-16, PMoF-18)



d Type 4 (PMaL-4, PVeL-5, PKaL-7, PSrL-10,PKkL-14)

e Type 5 (PKnL-12)



f. Type 6 (PVaL-15, PPaF 17)

4.6 1.4 Coconut water agar

All isolates produced white sparse aerial mycelium, stellate at the center and radiating from center to periphery The margin of the colony was irregular (Plate 15C)

4.6 1 5 V8 juice agar

On V8 juice agar, all *Phytophthora* isolates produced appressed white aerial mycelium with weak rosette growth pattern The edges of the colony were well defined (Plate 15D)

It is also observed from the Table 12 that, the colony characters of the pathogen varied among the different media, but variation among the isolates was noticed only in carrot agar medium

4.6.2 Growth rate

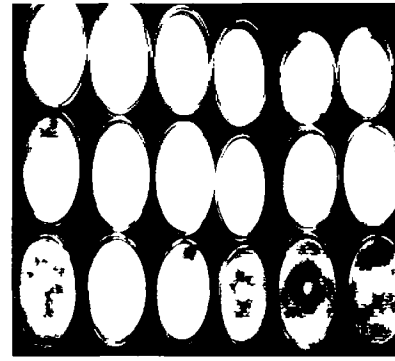
Growth rate of 18 isolates of pathogen on different media was studied in 9 cm sized Petri plate and results are presented in, Tables 13 – 17, which revealed that, all the five media were differing from each other in supporting the growth of the pathogen

On carrot agar medium, a slight variation was observed among isolates in colony diameter, which ranged from 1 7 to 2 5 cm at first day after inoculation Diameter of the colony recorded at 4th day after inoculation also showed a variation of 7 5 to 9 cm It is also observed that, isolate PTuL-11 was very fast growing and attained full growth at 4 DAI, whereas, PKoL 2,PVeL 5, PKnL-12 and PDeL-13 were slow growers which have taken 6 days for the full growth Growth rate also found to be varied from 1 9 to 2 3 cm among the isolates with maximum in PTuL-11 and minimum (1 9 cm) for PKoL-2 (Table 13)

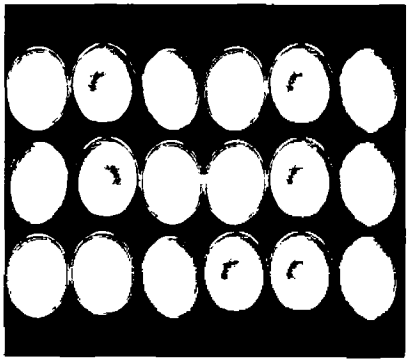
From the Table 14, it is noted that, the colony diameter recorded at first day after inoculation on PDA showed variation among the isolates ranged from 1 5 to 2 8 cm But not much difference was observed thereafter and full growth

Plate 15. Cultural characters

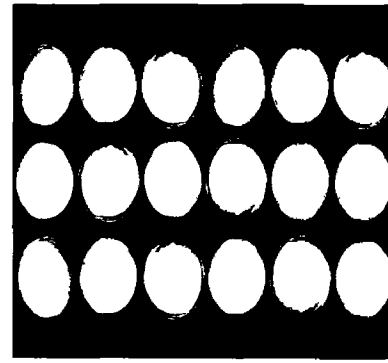
A. Potato dextrose agar medium



B. Oat meal agar medium



C. Coconut water agar



D. V8 juice agar

Table 13.Growth rate of *Phytophthora* isolates of nutmeg on carrot agar medium

Isolate	Mean diameter of colony (cm)						Days for full growth	Growth rate (cm)
	Days after incubation							
	1	2	3	4	5	6		
PPaL-1	21	42	69	88	90		5	22
PKoL 2	18	41	62	75	84	90	6	19
PPoL 3	18	39	62	79	90		5	20
PMaL 4	22	41	72	88	90		5	22
PVeL-5	19	41	60	80	85	90	6	20
PKtL-6	19	39	62	85	90		5	22
PKaL-7	23	48	79	88	90	-	5	22
PMtL 8	20	43	62	84	90	-	5	21
PThL-9	21	44	69	85	90	-	5	21
PSrL 10	25	50	76	88	90		5	21
PTuL-11	20	42	64	90	-	-	4	23
PKnL 12	22	43	63	81	87	90	6	20
PDeL 13	17	38	48	80	87	90	6	21
PKkL-14	18	39	62	83	90		5	20
PVaL-15	23	42	61	83	90		5	20
PKuL 16	20	42	69	88	90	-	5	22
PPaF-17	20	40	64	80	90		5	20
PMoF 18	20	42	61	84	90	-	5	21

Table 14 Growth rate of *Phytophthora* isolates of nutmeg on potato dextrose agar medium

Isolate	Mean diameter of colony (cm)							Days for full growth	Growth rate (cm)
	Days after incubation								
	1	2	3	4	5	6	7		
PPaL 1	28	41	57	73	84	90		6	18
PKoL-2	15	36	53	64	71	83	90	7	16
PPoL-3	21	43	63	79	90			5	20
PMaL 4	16	36	53	72	79	85	90	7	19
PVeL 5	19	40	56	71	83	90		6	17
PKtL 6	18	41	57	78	87	90	-	6	19
PKaL-7	15	37	50	61	73	82	90	7	15
PMtL-8	18	38	56	71	79	86	90	7	18
PThL 9	20	41	62	81	90			5	20
PSrL 10	17	37	46	77	86	90	-	6	19
PTuL 11	13	35	51	64	72	81	90	7	17
PKnL 12	15	35	53	74	80	86	90	7	20
PDeL-13	19	39	56	75	84	90		6	19
PKkL-14	17	35	57	76	86	90		6	20
PVaL 15	14	36	49	66	75	83	90	7	17
PKuL 16	18	39	54	73	84	90		6	18
PPaF 17	18	37	52	69	74	84	90	7	15
PMoF 18	16	35	51	62	72	83	90	7	15

was noticed in 5 7 days The isolates PPoL 3 and PThL-9 showed full growth at 5 DAI with a maximum growth rate of 2 cm, and minimum growth rate (1 5 cm) was in PKaL-7, PPaF-17 and PMoF-18

On oat meal agar medium, not much variation among the isolates was noticed with respect to colony diameter, full growth and growth rate (Table 15) The growth of the pathogen was faster as compared to other media and 72 per cent isolates showed full growth at 4 DAI and the growth rate of the isolates were 2 1 to 2 3 cm

On coconut water agar medium, the colony diameter of the isolates was within the range of 1 1 to 1 7 cm at one day after inoculation The isolates PPoL-3, PKaL 7 and PThL 9 recorded full growth at 6th day of inoculation and the growth rate ranged from 1 2 1 9 cm with maximum in PKaL-7 followed by PPoL-3 (1 8 cm) (Table 16)

From the Table 17, it is evident that, in V8 juice agar medium, the growth of the isolates were comparatively slow and required 7 9 days for the full growth and the growth rate was very less, ranged from, 0 8 – 1 3 cm

From the data presented in Table 18, it is observed that, there is variation among the days for full growth and growth rate on different media Among the different media tested, maximum growth rate (2 2 cm) was observed in oat meal agar followed by carrot agar (2 1 cm), potato dextrose agar (1 8 cm), coconut water agar (1 6 cm) and V8 juice agar (1 1cm) Minimum days (4-5) for full growth were also observed in oat meal agar medium followed by carrot agar with 4 6 days V8 juice medium showed maximum days (7-9) for the full growth in 9 cm sized dish

On summarizing the results, it is evident that, slight variation exists in the growth rate, among the various isolates in different media Of the different media tested, oat meal agar was found to be the best one in promoting the growth of

Table 15. Growth rate of *Phytophthora* isolates of nutmeg on oat meal agar medium

Isolate	Mean diameter of colony (cm)					Days for full growth	Growth rate (cm)
	Days after incubation						
	1	2	3	4	5		
PPaL-1	2 4	4 6	7 7	9 0	-	4	2 2
PKoL-2	2 4	4 8	7 2	9 0		4	2 1
PPoL 3	2 5	5 0	7 4	9 0		4	2 2
PMaL 4	2 1	4 4	6 6	8 6	9 0	5	2 2
PVeL-5	2 1	4 5	6 6	9 0	-	4	2 3
PKtL 6	2 1	4 3	6 8	8 6	9 0	5	2 2
PKaL 7	2 6	5 0	7 8	9 0		4	2 1
PMtL 8	2 4	4 6	7 7	9 0		4	2 2
PThL-9	2 4	4 8	7 3	9 0		4	2 2
PSrL-10	2 6	5 2	7 7	9 0		4	2 1
PTuL-11	2 0	4 2	6 9	8 7	9 0	5	2 3
PKnL 12	2 0	4 1	6 5	8 4	9 0	5	2 1
PDeL 13	2 7	5 0	7 9	9 0		4	2 1
PKkL 14	2 3	4 5	7 6	9 0		4	2 2
PVaL 15	2 2	4 4	6 9	8 7	9 0	5	2 1
PKuL-16	2 6	4 7	7 0	9 0		4	2 1
PPaF 17	2 3	4 9	7 6	9 0		4	2 2
PMoF 18	2 4	4 9	7 1	9 0		4	2 2

Table 16 Growth rate of *Phytophthoa* isolates of nutmeg on coconut water agar medium

Isolate	Mean diameter of colony (cm)								Days for full growth	Growth rate (cm)
	Days after incubation									
	1	2	3	4	5	6	7	8		
PPaL 1	1 5	2 6	3 8	5 6	6 8	8 1	9 0	-	7	1 4
PKoL 2	1 3	2 1	3 5	4 9	6 1	7 2	8 4	9 0	8	1 2
PPoL-3	1 7	2 7	4 0	7 1	8 3	9 0			6	1 8
PMaL 4	1 3	2 2	3 5	5 1	6 4	7 6	8 3	9 0	8	1 3
PVeL 5	1 5	2 5	3 7	5 5	6 8	8 4	9 0	-	7	1 3
PKtL 6	1 5	2 7	3 8	5 6	7 3	8 2	9 0	-	7	1 4
PKaL-7	1 7	2 8	4 1	7 3	8 4	9 0			6	1 9
PMtL-8	1 5	2 5	4 0	6 2	7 4	8 2	9 0		7	1 6
PThL 9	1 6	2 7	3 9	5 4	7 9	9 0			6	1 3
PSrL 10	1 4	2 6	4 0	6 1	7 5	8 3	9 0		7	1 6
PTuL 11	1 1	2 2	3 6	4 9	6 2	7 4	8 5	9 0	8	1 3
PKnL-12	1 3	2 3	3 6	5 3	6 7	7 6	8 7	9 0	8	1 3
PDeL 13	1 5	2 6	4 0	6 4	7 6	8 3	9 0		7	1 6
PKkL 14	1 4	2 6	3 7	5 8	6 7	8 2	9 0		7	1 5
PVaL 15	1 2	2 3	3 5	5 0	6 1	7 3	8 4	9 0	8	1 3
PKuL 16	1 6	2 7	3 8	5 2	6 6	8 1	9 0		7	1 2
PPaF 17	1 3	2 3	3 6	5 2	6 2	7 5	8 4	9 0	8	1 3
PMoF-18	1 2	2 1	3 4	5 2	6 4	7 7	8 5	9 0	8	1 3

Table 17. Growth rate of *Phytophthora* isolates of nutmeg on V8 juice agar medium

Isolate	Mean diameter of colony (cm)									Days for full growth	Growth rate (cm)
	Days after incubation										
	1	2	3	4	5	6	7	8	9		
PPaL 1	0 9	1 5	2 3	3 5	5 4	6 3	7 4	8 2	9 0	9	0 8
PKoL 2	1 0	1 8	3	4 1	6 5	7 6	8 3	9 0		8	1 0
PPoL 3	1 2	2 0	3 4	4 5	7 2	8 4	9 0			7	1 1
PMaL-4	1 0	1 6	2 4	3 6	5 9	6 8	7 9	8 3	9 0	9	0 9
PVeL 5	1 2	2 1	3 4	4 7	6 5	8 1	9 0	-	-	7	1 2
PKtL 6	1 0	1 9	2 8	4 1	6 3	7 6	8 5	9 0		8	1 0
PKaL 7	1 0	1 6	2 8	3 4	5 7	7 5	8 4	9 0		8	0 9
PMtL-8	1 0	1 8	2 9	3 9	6 1	7 8	8 7	9 0		8	1 0
PThL 9	1 0	2 0	3 1	5 1	6 7	7 9	8 6	9 0	-	8	1 4
PSrL 10	1 3	2 1	3 5	4 7	7 2	8 0	9 0			7	1 1
PTuL 11	1 2	2 0	3 3	4 5	7 5	8 3	9 0		-	7	1 1
PKnL-12	0 9	1 6	2 5	3 7	5 6	6 9	7 8	8 4	9 0	9	0 9
PDeL-13	1 2	2 1	3 5	5 3	7 4	8 5	9 0			7	1 3
PKkL 14	1 0	1 8	2 8	4 2	6 5	7 7	8 5	9 0	-	8	1 1
PVaL-15	1 3	2 1	3 3	4 5	7 2	8 1	9 0			7	1 0
PKuL 16	1 3	2 1	3 5	4 8	7 5	8 4	9 0			7	1 2
PPaF 17	1 0	2 0	3 2	4 6	6 9	7 4	8 3	9 0		8	1 2
PMoF-18	1 2	2 0	3 3	4 4	7 3	8 2	9 0	-	-	7	1 1

Table 18.Growth rate of *Phytophthora* isolates of nutmeg on different media

Media	Days for full growth (9 cm Petridish)	Average growth rate (cm)
Carrot Dextrose Agar	4 – 6	2 1
Potato Dextrose Agar	5 -7	1 8
Oat meal Agar	4 – 5	2 2
Coconut water Agar	6 – 8	1 6
V8 juice Agar	7 – 9	1 1

the pathogen followed by carrot agar Minimum growth rate was found in V8 juice agar medium

Comparison on the cultural characters of *Phytophthora* isolates of nutmeg with other *Phytophthora* spp such as *P meadii*, *P palmivora*, *P capsici*, in *P colocasiae*, *P citrophthora* and *P ramorum* on carrot agar medium are presented in Table 19 The different growth patterns of *Phytophthora* isolates of nutmeg observed in the present study were distinct/weak rosette, concentric ring, lobbed and powdery Weak rosette and concentric ring like growth patterns were observed in *P meadii* and *P ramorum* on carrot agar medium None of the characters were found similar to *P capsici* and *P colocasiae* It is revealed that, cultural characters of *Phytophthora* isolates of nutmeg showed much similarity to *P meadii* and *P ramorum* with respect to the weak rosette and concentric ring like colony patterns

4 6 3 Morphological characters

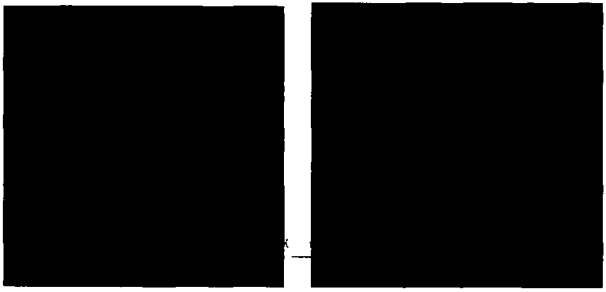
Morphological characters such as type of mycelium, sporangial shape, size, L/B ratio and pedicel length, type of papillae, type of sporangiophore, chlamydospores and sexual structures of various isolates of the pathogen in different media were recorded The mycelium was coenocytic, hyaline and branched The sporangia were borne either terminally or laterally on the sporangiosphore in a simple sympodial fashion and were caduceus (Plate 16A 1&2) All isolates produced semipapillate sporangia, with diameter of 4 2 to 8 0 µm Sporangia were mostly ovoid/ elongated –ovoid/ ellipsoid, with round base Variation in sporangial shape was observed with those from culture media and the host The sporangia of PMoF-18 from the culture medium showed typical shape as observed in other isolates, whereas, the sporangia from the host showed elongated, cylindrical and ovoid with bend-beak (Plate 16B) Oil globules were observed in all isolates

Table 19. Comparison of cultural characters of different *Phytophthora* isolates of nutmeg with other *Phytophthora* spp. in carrot agar medium

Characters	*Phytophthora* sp of nutmeg	*P. meadii*	*P palmivora*	*P.capsici*	*P.colocasiae*	*P.citrophthora*	*P ramorum*
Mycelia	Dull white/ white/cottony white	White	Cottony white	Dull white	Dull white	White	White
Growth pattern	Distinct/ weak rosette, concentric rings, lobbed, powdery,	Weak rosette, concentric rings	Concentric rings	Weak stellate, faded	No distinct colony pattern	Weak rosette	Weak rosette, concentric rings

Plate 16. Sporangial characters

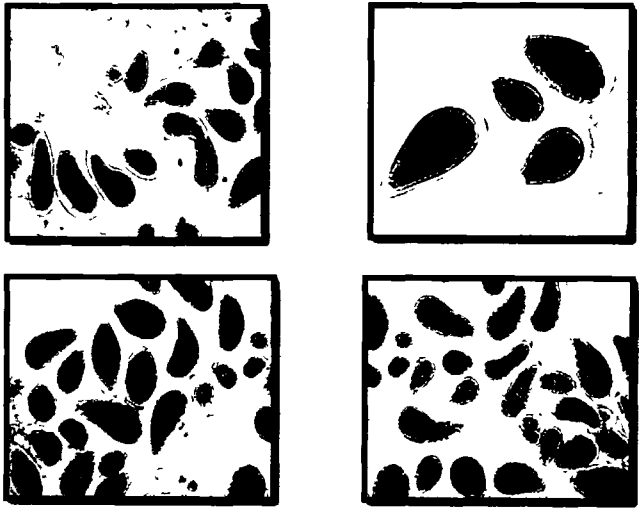
A. Sporangia from culture medium



1.Terminal sporangia

2.Lateral sporangia

B.Sporangia from host surface



All the isolates produced abundant sporangia in all the five media tested (Plate 17 - 21) However, time for sporangial production and sporangial count (per microscopic field) were influenced by different media (Table 20) In carrot agar medium, all isolates except PKuL-16, PPaF-17 and PMoF-18 produced sporangia in 24 h and maximum sporangial count was observed in PPaL-1 Seventy two per cent isolates showed sporangial production in 24 h in potato dextrose agar medium and maximum sporangial count (39) was observed with PMaL-4 In oat meal agar, sporangial production was late as compared to carrot agar and potato dextrose agar media Eighty three per cent of the isolates showed sporangial production only at 72 h and early sporangial production (48 h) was observed with isolate, PPaL-1, PKoL-2 and PPoL-3 and the maximum sporangial count (46) was recorded in PPaL-1 In coconut water agar medium, all isolates recorded sporangial production at 48 h with maximum (35) in PPoL 3 In V8 juice agar 89 per cent isolates showed sporangial production only at 72 h and two isolates PKtL-6 and PTuL-11 showed early production at 48 h with maximum (27) in PDeL-13

It was evident from the above data that, there is variation in time for sporangial production and count among the isolates and different media tested Early sporangial production (24 – 48 h) was observed in carrot agar and potato dextrose agar media whereas sporangial count was high in oat meal agar and carrot agar media It is also noted that, sporangial count was less with PMoF-18 (an isolate from fruit) in all the five media tested

4.6.3.1 Sporangial size

Variation in sporangial length was noticed among the media, but any noticeable variation was observed with respect to sporangial breadth and L/B ratio (Table 21) However, variations in sporangial length and breadth were noticed among the isolates in different media Among the isolates, PMaL 4 recorded maximum length of 71 9 µm in oat meal agar and 49 1 µm in carrot agar and also the maximum breadth (28 7 µm) in V8 juice agar PVeL 5 showed

Plate 17. Sporangia of *Phytophthora* isolates of nutmeg

Carrot agar medium

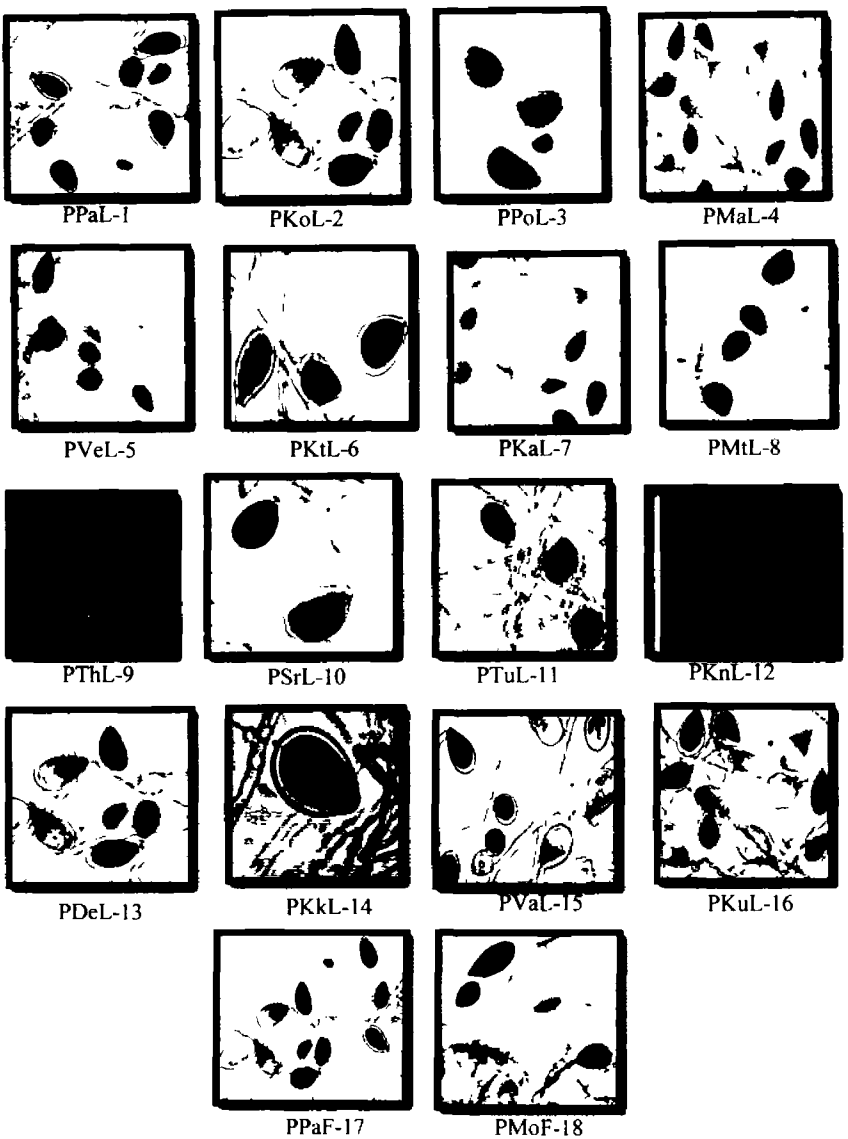


Plate 18. Sporangia of *Phytophthora* isolates of nutmeg

Potato dextrose agar medium

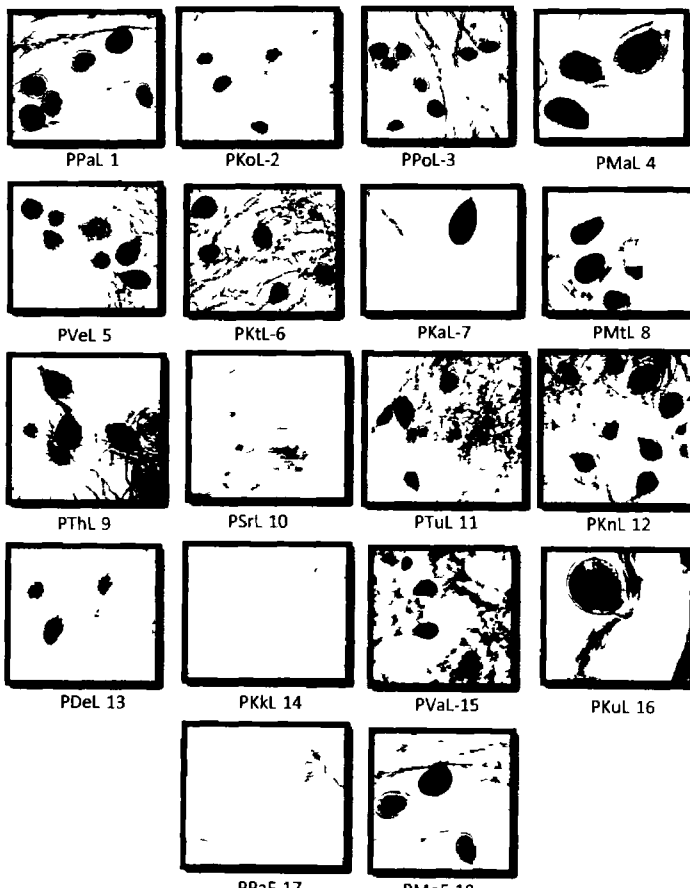


Plate 19. Sporangia of *Phytophthora* isolates of nutmeg

Oat meal agar medium

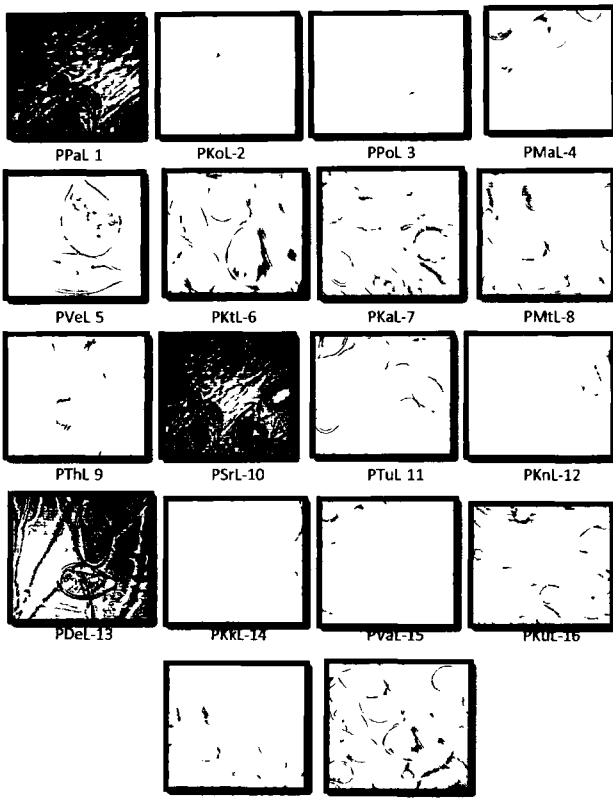


Plate 20. Sporangia of *Phytophthora* isolates of nutmeg

Coconut water agar medium

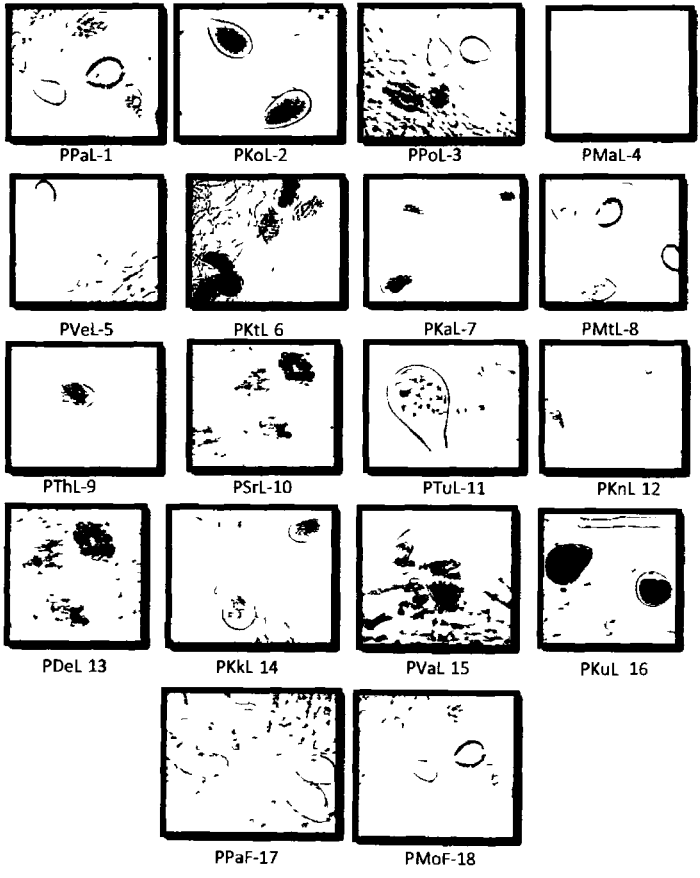


Plate 21. Sporangia of *Phytophthora* isolates of nutmeg

V8 juice agar medium

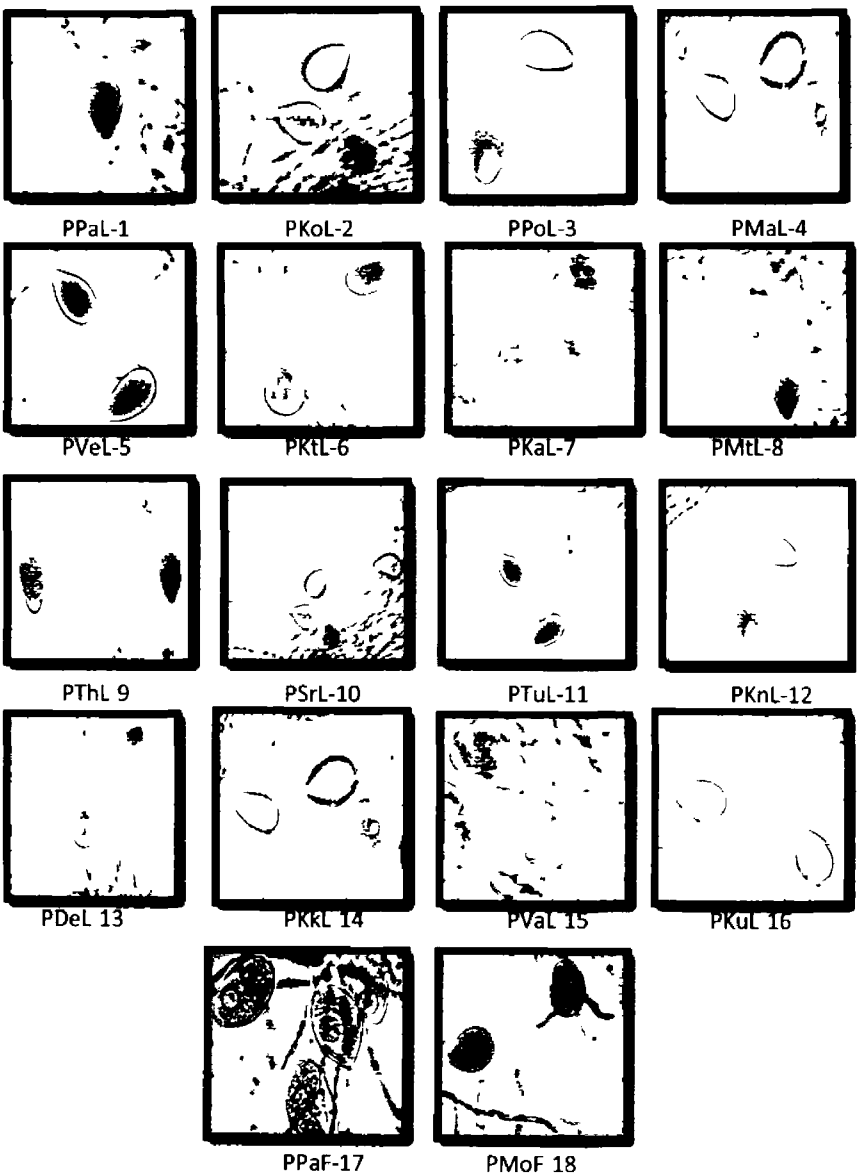


Table 20.Sporangial production in different media

Isolates	Carrot agar		Potato dextrose agar		Oat meal agar		Coconut water agar		V8 juice agar	
	Time for sporangial production (H)	*Sporangial count	Time for sporangial production (H)	*Sporangial count	Time for sporangial production (H)	*Sporangial count	Time for sporangial production (H)	*Sporangial count	Time for sporangial production (H)	*Sporangial count
PPaL-1	24	45	24	22	48	46	48	19	72	17
PKoL-2	24	37	24	20	48	27	48	20	72	19
PPoL-3	24	27	24	34	48	33	48	35	72	15
PMaL-4	24	43	24	39	72	42	48	31	72	20
PVeL-5	24	29	24	32	72	41	48	31	72	18
PKtL-6	24	37	24	20	72	29	48	34	48	23
PKaL-7	24	34	24	28	72	42	48	19	72	25
PMtL-8	24	27	48	31	72	31	48	26	72	20
PThL 9	24	39	24	18	72	40	48	16	72	22
PSrL 10	24	30	48	36	72	27	48	22	72	23
PTuL-11	24	41	24	23	72	39	48	30	48	13
PKnL-12	24	33	24	29	72	36	48	19	72	16
PDeL-13	24	29	24	33	72	27	48	24	72	27
PKkL-14	24	40	24	37	72	30	48	33	72	19
PVaL-15	24	31	24	25	72	41	48	28	72	18
PKuL-16	48	23	48	19	72	24	48	19	72	15
PPaF-17	48	23	48	22	72	25	48	23	72	17
PMoF-18	48	21	48	19	72	24	48	20	72	16

*Mean of three microscopic fields under 10 x 10 X

Table 21 Sporangial size of various *Phytophthora* isolates in different media

Isolates	*Sporangial dimensions (10 x 40 X)														
	Carrot agar			Potato dextrose agar			Oat meal agar			Coconut water agar			V8 juice agar		
	Length (µm)	Breadth (µm)	L/B ratio	Length (µm)	Breadth (µm)	L/B ratio	Length (µm)	Breadth (µm)	L/B ratio	Length (µm)	Breadth (µm)	L/B ratio	Length (µm)	Breadth (µm)	L/B ratio
PPaL-1	47 0	29 9	1 6	36 6	25 9	1 4	48 6	26 8	1 8	44 9	29 9	1 5	35 8	26 0	1 4
PKoL 2	47 5	26 0	1 8	20 9	16 4	1 3	35 6	20 6	1 7	37 9	24 8	1 5	37 9	24 7	1 5
PPoL-3	44 5	29 9	1 5	33 4	22 7	1 5	41 1	23 6	1 7	40 3	29 1	1 5	40 2	25 5	1 6
PMaL-4	49 1	29 0	1 7	30 1	16 8	1 2	71 9	30 2	1 7	35 1	24 5	1 4	39 8	28 7	1 4
PVeL-5	47 5	30 4	1 6	41 0	27 4	1 5	53 7	32 6	1 7	37 7	26 6	1 4	35 6	25 7	1 4
PKtL 6	36 6	22 9	1 6	22 2	19 0	1 2	36 0	20 8	1 7	40 8	30 5	1 3	33 2	25 9	1 3
PKaL-7	42 7	25 1	1 7	20 2	20 3	1 5	49 5	25 1	2 0	47 8	36 0	1 4	35 4	23 8	1 5
PMtL-8	43 4	26 9	1 6	32 4	21 1	1 5	51 9	32 4	2 2	33 8	22 1	1 5	28 4	20 7	1 7
PThL 9	41 5	26 8	1 6	40 8	22 1	1 4	54 9	29 5	1 9	47 8	31 9	1 5	31 7	22 0	1 4
PSrL 10	43 9	27 3	1 6	37 5	29 1	1 3	60 1	33 0	1 8	35 5	27 0	1 3	36 8	26 4	1 4
PTuL 11	38 8	24 9	1 6	31 7	23 1	1 4	54 3	29 1	1 9	38 5	25 9	1 5	30 0	19 7	1 4
PKnL-12	32 8	21 2	1 6	29 7	22 6	1 3	49 3	25 3	1 9	31 5	24 4	1 3	34 6	22 7	1 5
PDeL 13	37 2	22 6	1 7	28 6	21 0	1 4	45 6	28 5	1 6	38 6	26 7	1 5	40 1	26 2	1 5
PKkL-14	34 7	19 5	1 8	38 2	25 4	1 5	71 3	23 6	1 9	48 2	30 7	1 6	29 3	25 7	1 1
PVaL 15	46 5	30 3	1 4	32 2	22 1	1 5	36 5	34 8	1 5	41 3	28 1	1 5	30 1	22 4	1 3
PKuL 16	32 4	21 1	1 5	29 4	20 1	1 5	40 4	20 4	2 0	48 3	31 6	1 5	35 2	24 5	1 4
PPaF-17	37 4	23 6	1 6	30 2	21 0	1 4	48 3	31 6	1 5	35 4	26 3	1 4	42 9	25 7	1 5
PMoF-18	35 4	23 7	1 5	38 5	25 9	1 5	42 4	20 1	2 1	38 6	26 7	1 5	30 5	27 9	1 3

*Average of 10 sporangia

maximum length (41 0 µm) and breadth (27 4 µm) in PDA and breadth in carrot agar In coconut water agar, maximum length (48 3 µm) and breadth (36 0 µm) were recorded by PKuL-6 and PKaL-7 respectively and the isolate PPaF 17 showed maximum length in V8 juice agar medium Much variation was not noticed in L/B ratio among isolates

While considering the aspects of sporangial dimensions, media and the isolates together, it is noticed that, maximum length (71 9 µm), breadth (36 0 µm) and L/B ratio (2 2) were recorded by PMaL-4 in oat meal agar, PkaL-7 in coconut water agar and PMtL 8 in oat meal agar media respectively

Data presented in Table 22, showed variation in sporangial dimensions such as length breadth and L/B ratio, time for sporangial production, sporangial count and diameter of papilla in different media Maximum sporangial length (71 9 µm), range (35 6 - 71 9 µm), size (49 5 x 27 1 µm), L/B ratio (1 8), sporangial count (24 – 46) and diameter of papillae (8 0 µm) with range 5 2 – 8 0 µm were observed in oat meal agar followed by carrot agar medium Maximum sporangial width (36 0 µm) with a range of 22 1 – 36 0 µm was observed in coconut water agar followed by oat meal agar (20 1-34 8 µm) and carrot agar media (20 9-30 4 µm) Minimum sporangial length, breadth and L/B ratio were observed in potato dextrose agar medium Early sporangial production (24 – 48 h) was observed in carrot agar and potato dextrose agar media Eventhough, sporangial count was high in oat meal agar, sporangial production was late (48 72 h) in this medium

Considering all the parameters, oat meal agar and carrot agar media were found to be the best media for studying the morphological characters of *Phytophthora* spp

Table 22. Sporangial characters of *Phytophthora isolates* in different media

Media	Length range (µm)	Breadth range (µm)	Average size (µm)	L/B ratio		Time for sporangial production (H)	Sporangial count range (10 x 10 X)	Diameter of papilla (µm)
				Range	Average			
Carrot agar	32 4 - 49 1	20 9 30 4	41 x 25 6	1 4 1 8	1 6	24 48	21-45	5 6 – 7 8
Potato dextrose agar	20 2 - 41 0	16 4 27 4	31 9 x 22 3	1 2 1 5	1 4	24 48	18 39	4 2 - 6 8
Oat meal agar	35 6 - 71 9	20 1 34 8	49 5 x 27 1	1 5 - 2 2	1 8	48 72	24-46	5 2 8 0
Coconut water agar	31 5 - 48 3	22 1 36 0	40 1 x 27 9	1 3- 1 6	1 5	48	16-35	5 3 7 5
V8 juice agar	29 3 - 42 9	19 7 - 28 7	34 9 x 24 7	1 3 - 1 6	1 4	48 72	17 25	4 2 6 3

4.6 3.2 Pedicel length of sporangia

The pedicel length of 18 isolates varied from 10 21 to 20 24 µm and majority were in the range of 14 16 µm Maximum pedicel length was recorded in PKtL-6 and minimum in PKaL-7 (Table 23)

4.6 3 2 Chlamydospore production

From the data presented m Table 24, it was evident that, all isolates produced abundant chlamydospores in all the five media tested (Plate 22 26) They were globose, hyaline, mostly thin walled, and formed intercalary and terminally There was no variation with regard to the shape and position of chamydospores in different media However, slight variation was observed in chlamydospore count and diameter among the media and isolates Highest diameter and count was observed in carrot agar medium which ranged from 16 4 – 38 5 µm and 21- 42 no respectively of which, PMaL 4 showed highest diameter (38 5 µm) and PVaL-15 showed the highest count of 42 All other media were similar to each other with respect to size and count and slight variation was observed among the isolates

It was observed that, among different media tested, highest chlamydospores count and diameter were recorded in carrot agar medium It was also found that, isolate PVaL 15 showed highest chlamydospores production in all the media while isolate PMaL-4 recorded highest chamydospore diameter in all media except potato dextrose agar and V8 juice agar in which, PKnL 12 showed highest diameter

4.6.3.3 Sexual structures

Sexual organ, oogonium of the pathogen was observed on carrot agar medium (Plate 22 B)

Table 23. Pedicel length of different *Phytopthora* isolates on carrot agar medium

Isolate	Pedicel length (10 x 40 X)	
	Range (µm)	Average (µm)
PPaL-1	5 40 – 16 00	10 33
PKoL-2	8 64 – 18 34	14 66
PPoL-3	7 86 – 21 30	15 17
PMaL-4	6 29 - 23 46	16 77
PVeL-5	5 84 – 21 72	15 90
PKtL-6	18 46 – 24 53	20 24
PKaL-7	5 70 – 14 26	10 21
PMtL-8	7 32 – 21 56	15 03
PThL-9	8 42 – 20 40	15 39
PSrL-10	5 82 – 21 41	14 82
PTuL-11	6 23 – 22 34	15 61
PKnL-12	8 21 – 23 20	16 31
PDeL-13	6 10 – 23 31	16 27
PKkL-14	5 70 – 20 40	15 54
PVaL-15	8 26 – 19 50	14 59
PKuL-16	6 32 – 21 49	16 68
PPaF-17	5 91 – 16 40	10 42
PMoF-18	7 60 – 15 38	11 35

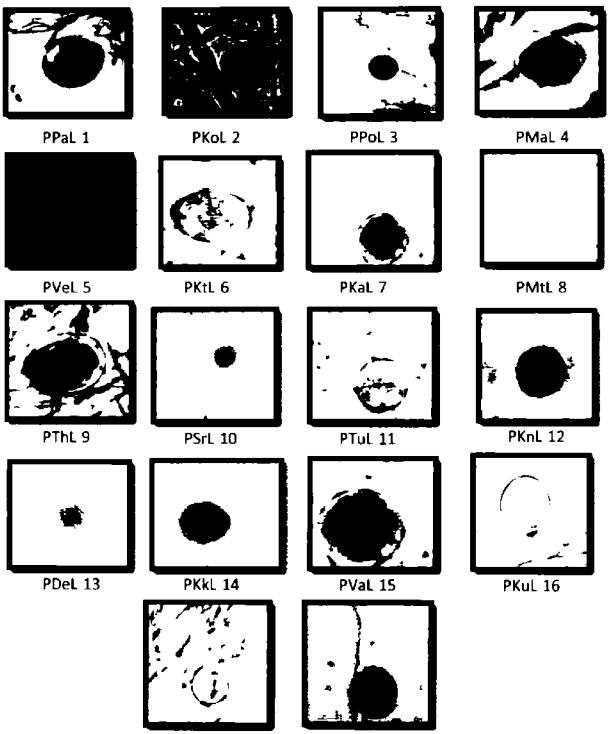
Table 24 Chlamydospore production of various *Phytophthora* isolates in different media

Isolates	Carrot agar		Potato dextrose agar		Oat meal agar		Coconut water agar		V8 juice agar	
	Diameter (µm)	*Chlamydospore count	Diameter (µm)	*Chlamydospore count	Diameter (µm)	*Chlamydospore count	Diameter (µm)	*Chlamydospore count	Diameter (µm)	*Chlamydospore count
PPaL-1	22 2	25	25 2	18	21 7	21	34 2	15	23 4	14
PKoL-2	34 9	37	21 1	21	20 3	18	30 1	19	20 5	10
PPoL 3	21 1	29	25 3	14	24 4	23	21 7	14	22 6	18
PMaL-4	38 5	24	21 1	19	26 0	21	35 8	21	22 1	15
PVeL-5	20 2	21	20 8	18	17 5	14	23 4	21	19 3	14
PKtL-6	34 6	37	26 0	18	22 6	25	32 1	32	21 7	19
PKaL-7	27 3	26	24 4	23	21 2	21	28 1	23	20 3	21
PMtL-8	19 8	38	16 2	21	19 7	28	20 8	27	18 8	21
PThL-9	28 0	33	23 5	14	16 9	24	20 0	21	23 6	15
PSrL 10	17 6	24	20 0	18	18 4	16	19 9	18	21 5	17
PTuL-11	29 8	24	21 0	21	21 6	21	23 1	15	17 5	23
PKnL-12	30 2	35	34 4	24	20 9	19	20 5	23	23 9	21
PDeL-13	24 7	37	19 7	18	22 8	26	23 7	25	16 4	23
PKkL-14	29 8	30	21 5	16	20 4	14	23 6	19	23 8	18
PVaL 15	36 4	42	27 4	27	20 1	28	31 5	32	17 0	23
PKuL 16	32 0	26	23 0	21	25 8	19	17 5	15	16 8	19
PPaF-17	16 4	21	22 7	18	21 1	21	26 7	18	16 6	14
PMoF-18	17 2	28	20 4	16	22 9	18	21 2	23	15 3	18

*Mean of three microscopic fields at 10 x 10 X

Plate 22. A. Chlamydospores of various *Phytophthora* isolates

Carrot agar medium



B. Oogonium of the pathogen

PMaL 4

Plate 23. Chlamydospores of various *Phytophthora* isolates

Potato dextose agar medium

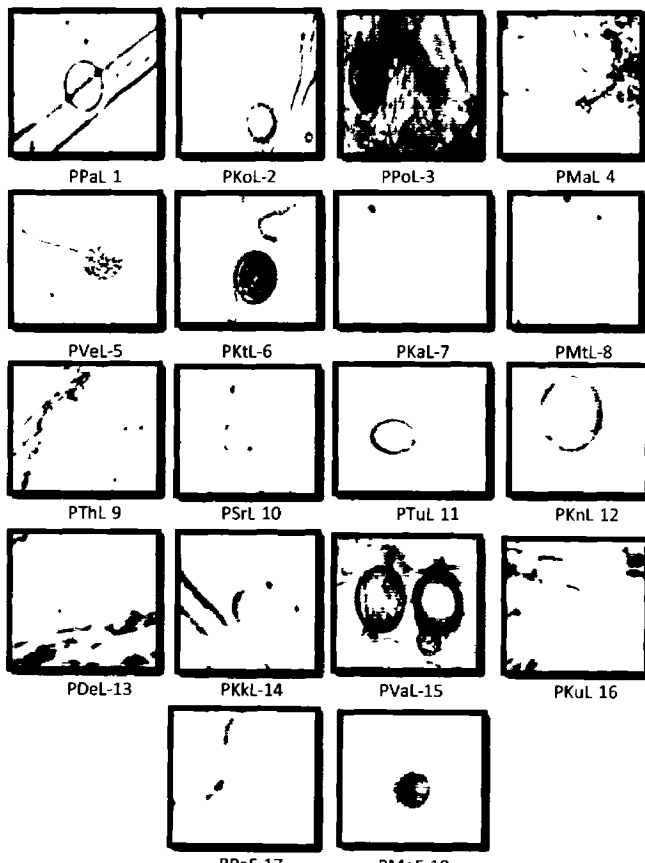


Plate 24. Chlamydospores of various *Phytophthora* isolates

Oat meal agar medium

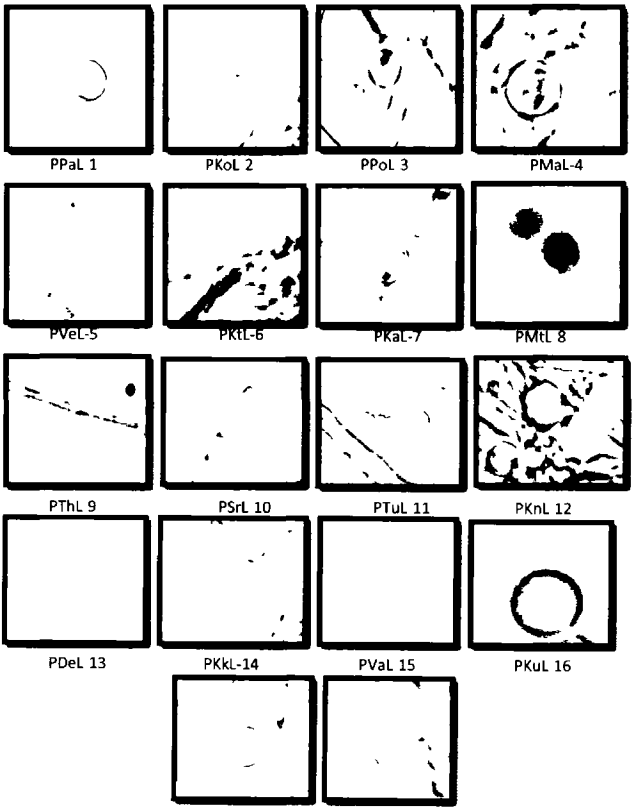


Plate 25. Chlamydospores of various *Phytophthora* isolates

Coconut water agar medium

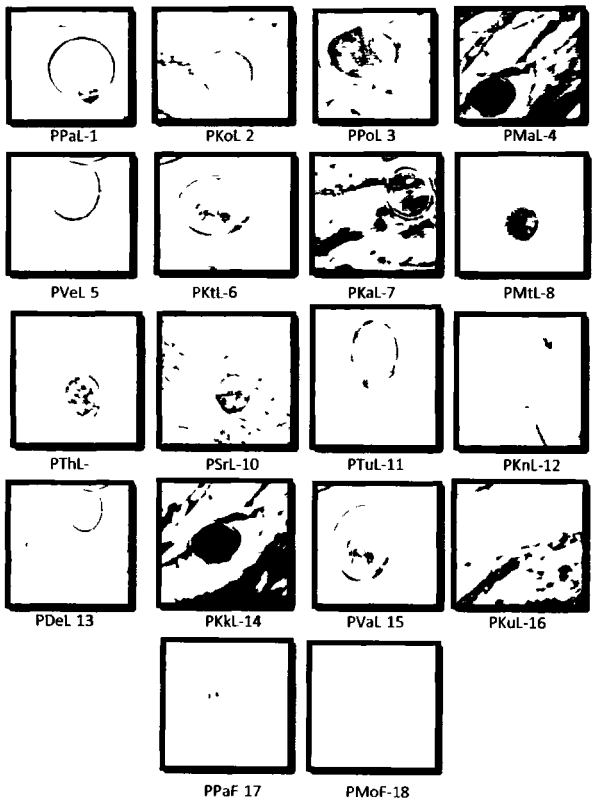
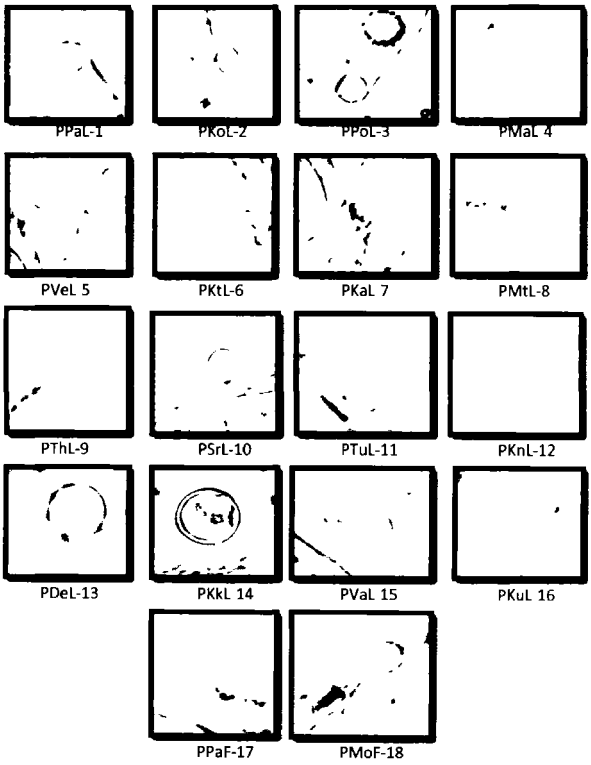


Plate 26. Chlamydospores of various *Phytophthora* isolates

V8 juice agar medium



Summarising the morphological characters of the pathogen, the mycelium was coenocytic, hyaline and branched Sporangia were borne terminally /laterally on the sporangiophore in sympodial fashion and were caducous, semi papillate, ovoid/ elongated - ovoid/ ellipsoid in shape, sporangial length 20 2 – 71 9 µm, breadth 16 4 -36 0 µm, L/B ratio 1 4 – 1 8, average size 31 9-49 5 x 22 3 – 27 9 µm, pedicel length 10 21 – 20 24 µm and abundant chlamydospores with diameter ranged from 16-38 µm

Comparison of morphological characters of *Phytophthora* isolates of nutmeg with other *Phytophthora* spp such as *P meadii*, *P palmivora*, *P capsici*, *P colocasiae* *P citrophthora* and *P ramorum* are presented in Table 25 Considering the various morphological parameters, *Phytophthora* isolates of nutmeg were found to be different from *P palmivora* *P capsici* and *P citrophthora* and showed certain similarity with *P meadii* *P colocasiae* and *P ramorum* Morphologically, *Phytophthora* isolates of nutmeg were characterized by ovoid, ellipsoid, elongated ovoid, caducous semi papillate sporangia with abundant production of chlamydospores and in these respects, they resemble *P ramorum* and *P colocasiae* However, sporangial length, breadth, L/B ratio and pedicel length showed close similarity to *P meadii* It is also observed that, chlamydospore characters of the isolates such as presence, shape and diameter were more similar with *P colocasiae* but dissimilar to *P meadii* as chlamydospore production is rare in this species

Phytophthora isolates obtained from nutmeg plants could not be completely fitted into the morphological description of any of these known *Phytophthora* species eventhough they showed some similarity to *P meadii*, *P colocasiae* and *P ramorum*

Table 25. Comparison of morphological characters of *Phytophthora* isolates of nutmeg with other *Phytophthora* spp.

Morphological characters		*Phytophthora* isolates of nutmeg	*P meadii*	*P palmivora*	*P capsici*	*P colocasiae*	*P citrophthora*	*P ramorum*
Sporangial characters	Papilla	Semi papillate	Papillate	Papillate	Papillate	Semi papillate	Papillate	Semi papillate
	Caducous	Caducous	Caducous	Caducous	Caducous	Caducous	Non caducous	Caducous
	Shape	Elongated-ovoid/ellipsoid/ ovoid with round base	Ellipsoid/ elongated/ obpyriform	Elliptical to ovoid	Subspherical/ ovoid/ obovoid/ellipsoid/fusiform /pyriform	Ovoid/ ellipsoid	Ellipsoid/ broadly ovoid/ globose/ limoniform	Ellipsoid/ elongated-ovoid with round base
	Length (µm)	32 4 - 49 1	20 - 44	40 - 60	32 8 - 65 8	40 – 70	27 - 65 3	45 6 - 65
	Breadth (µm)	20 9 - 30 4	16 - 29	25 - 35	17 4 - 38 7	17 – 28	18 9 - 40 4	21 2 – 28 3
	L/B ratio	1 4 - 1 8	1 3 - 2 0	1 4 - 2 0	1 6 - 2 2	1 9 - 2 5	1 3 - 1 8	1 8 - 2 4
	Pedicel length (µm)	10 2 - 20 2	10 - 20	< 5	35 - 138	3 5 - 10	No pedicel	1 - 5
Chlamydospore	Presence	Abundant	Rare	Abundant	Abundant	Abundant	Rare	Abundant
	shape	Globose	Globose	Globose to subglobose	Globose to subglobose	Globose	Globose	Globose
	Diameter (µm)	16 - 38	16 - 30	32 - 42	28 - 29	17 - 38	25 – 35	46 - 60

4.7 Molecular characters

4.7 1 Genomic DNA isolation from *Phytophthora* isolates

Genomic DNA was extracted from the 18 *Phytophthora* isolates using the GenElute Plant Genomic DNA Mimprep Kit (SIGMA) Agarose gel electrophoresis of genomic DNA revealed the presence of intact bands in all the isolates indicating good quality DNA free from RNA

4.7.2 Amplification of the ITS region

PCR amplification of the ITS region was performed using primers ITS1 and ITS4 PCR products from the 18 *Phytophthora* isolates contained a single band and size of the amplified product was nearly 900 bp

4 7 3 Nucleotide sequence analysis

4.7 3.1 Nucleotide Blast

Homology search of nucleotide sequences obtained from 18 *Phytophthora* isolates with other reported *Phytophthora* sequences were carried out and the data were presented in Table 26 Of the 18, 11 isolates *viz* PPaL-1, PKoL 2, PVeL 5, PKtL-6, PKaL 7, PMtL 8, PThL-9, PTuL-11, PKnL-12, PPaF-17 and PMoF-18 showed maximum homology with *P colocasiae* and *P citrophthora* Whereas the isolates PPoL-3 and PVaL 15 showed maximum homology with *P meadii* followed by *P colocasiae* and PDeL 13, PKkL-14 and PKuL-16 showed maximum homology with *P colocasiae* followed by *P meadii* While PMaL 4 showed homology with *P meadii* followed by *P botryosa* and *P colocasiae* respectively Likewise, PSrL-10 showed maximum homology with *P colocasiae* followed by *P meadii* and *P botryosa* gene sequences in NCBI databank

4 7.3.2 Phylogenic analysis

Multiple sequence analysis was done with all 18 isolates by using ClustalW tool to find out relationship among the isolates Phylogenic tree showed that all the 18 isolates shared a common ancestor

Table 26 ITS-1 sequence analysis of *Phytophthora* isolates

Isolates	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e value
	Accession no	Name				
PPaL-1	JN661144 1	*Phytophthora colocasiae*	1524	99	99	0 0
	JN661147 1	*Phytophthora colocasiae*	1520	100	99	0 0
	JN661146 1	*Phytophthora colocasiae*	1520	100	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1520	100	99	0 0
	JN661141 1	*Phytophthora colocasiae*	1520	100	99	0 0
	JN661140 1	*Phytophthora colocasiae*	1520	100	99	0 0
	GU111605 1	*Phytophthora colocasiae*	1520	100	99	0 0
	JN661142 1	*Phytophthora colocasiae*	1519	100	99	0 0
	GU111603 1	*Phytophthora citrophthora*	1515	100	99	0 0
	GU111602 1	*Phytophthora citrophthora*	1515	100	99	0 0
PKoL 2	JN661146 1	*Phytophthora colocasiae*	1548	99	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1546	99	99	0 0
	GU111605 1	*Phytophthora colocasiae*	1546	99	99	0 0
	JN661147 1	*Phytophthora colocasiae*	1543	99	99	0 0
	GU111604 1	*Phytophthora colocasiae*	1541	99	99	0 0
	GU111602 1	*Phytophthora citrophthora*	1541	99	99	0 0
	GU111600 1	*Phytophthora citrophthora*	1539	99	99	0 0
	GU133066 1	*Phytophthora citrophthora*	1539	99	99	0 0
	JN661138 1	*Phytophthora colocasiae*	1535	99	99	0 0
	GU111602 1	*Phytophthora citrophthora*	1535	99	99	0 0
PPoL 3	JX155793 1	*Phytophthora meadii*	1120	99	94	0 0
	JN315692 1	*Phytophthora colocasiae*	1114	99	93	0 0
	AB367509 1	*Phytophthora meadii*	1114	100	93	0 0
	GU259352 1	*Phytophthora meadii*	1114	100	93	0 0
	FJ801908 1	*Phytophthora meadii*	1114	100	93	0 0
	JN661144 1	*Phytophthora colocasiae*	1110	100	93	0 0
	GU259325 1	*Phytophthora meadii*	1109	99	93	0 0
	GU259005 1	*Phytophthora colocasiae*	1109	99	93	0 0
	FJ801306 1	*Phytophthora meadii*	1109	99	93	0 0
	JN618809 1	*Phytophthora meadii*	1107	99	93	0 0

Isolates	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e value
	Accession no	Name				
PMaL-4	AB367509 1	*Phytophthora meadii*	1338	99	98	0 0
	GU259352 1	*Phytophthora meadii*	1338	99	98	0 0
	JN618705 1	*Phytophthora botryosa*	1334	99	98	0 0
	JN661144 1	*Phytophthora colocasiae*	1334	100	98	0 0
	FJ801306 1	*Phytophthora meadii*	1334	99	98	0 0
	GU983652 1	*Phytophthora meadii*	1332	99	98	0 0
	GU259325 1	*Phytophthora meadii*	1332	99	98	0 0
	GU259005 1	*Phytophthora colocasiae*	1332	99	98	0 0
	JN618809 1	*Phytophthora meadii*	1330	99	98	0 0
	JN618738 1	*Phytophthora meadii*	1328	99	98	0 0
PVeL-5	GU111605 1	*Phytophthora colocasiae*	1543	93	99	0 0
	JN661146 1	*Phytophthora colocasiae*	1541	91	99	0 0
	JN661141 1	*Phytophthora colocasiae*	1541	92	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1539	91	99	0 0
	GU111603 1	*Phytophthora citrophthora*	1539	93	99	0 0
	GU111604 1	*Phytophthora colocasiae*	1537	93	99	0 0
	GU111602 1	*Phytophthora citrophthora*	1537	93	99	0 0
	GU111601 1	*Phytophthora citrophthora*	1537	93	99	0 0
	GU111600 1	*Phytophthora citrophthora*	1537	93	99	0 0
	JN661147 1	*Phytophthora colocasiae*	1535	91	99	0 0
PKtL-6	JN661147 1	*Phytophthora colocasiae*	1443	100	99	0 0
	JN661146 1	*Phytophthora colocasiae*	1443	100	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1443	100	99	0 0
	JN661140 1	*Phytophthora colocasiae*	1433	100	99	0 0
	JN661138 1	*Phytophthora colocasiae*	1433	100	99	0 0
	GU111605 1	*Phytophthora colocasiae*	1433	100	99	0 0
	JN661145 1	*Phytophthora colocasiae*	1437	100	99	0 0
	GU111604 1	*Phytophthora colocasiae*	1437	100	99	0 0
	GU111603 1	*Phytophthora citrophthora*	1437	100	99	0 0
	GU111602 1	*Phytophthora citrophthora*	1437	100	99	0 0

Isolates	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e value
	Accession no.	Name				
PKaL-7	JN661147 1	*Phytophthora colocasiae*	1461	100	99	0 0
	JN661146 1	*Phytophthora colocasiae*	1461	100	99	0 0
	JN661145 1	*Phytophthora colocasiae*	1461	100	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1461	100	99	0 0
	JN661138 1	*Phytophthora colocasiae*	1461	100	99	0 0
	GU111605 1	*Phytophthora colocasiae*	1461	100	99	0 0
	GU111604 1	*Phytophthora colocasiae*	1456	100	99	0 0
	GU111603 1	*Phytophthora citrophthora*	1456	100	99	0 0
	GU111601 1	*Phytophthora citrophthora*	1452	100	99	0 0
	GU133063 1	*Phytophthora citrophthora*	1452	100	99	0 0
PMtL-8	JN661146 1	*Phytophthora colocasiae*	1548	99	99	0 0
	JN661147 1	*Phytophthora colocasiae*	1546	99	99	0 0
	GU111605 1	*Phytophthora colocasiae*	1546	99	99	0 0
	JN661147 1	*Phytophthora colocasiae*	1543	99	99	0 0
	GU111609 1	*Phytophthora colocasiae*	1541	99	99	0 0
	GU111603 1	*Phytophthora citrophthora*	1541	99	99	0 0
	GU111601 1	*Phytophthora citrophthora*	1539	99	99	0 0
	GU111603 1	*Phytophthora citrophthora*	1537	99	99	0 0
	JN661138 1	*Phytophthora colocasiae*	1535	99	99	0 0
	GU111602 1	*Phytophthora citrophthora*	1535	99	99	0 0
PThL-9	JN661147 1	*Phytophthora colocasiae*	1387	100	97	0 0
	JN661146 1	*Phytophthora colocasiae*	1387	100	97	0 0
	JN661139 1	*Phytophthora colocasiae*	1387	100	97	0 0
	JN661138 1	*Phytophthora colocasiae*	1387	100	97	0 0
	JN661138 1	*Phytophthora colocasiae*	1387	100	97	0 0
	JN661140 1	*Phytophthora colocasiae*	1386	100	97	0 0
	GU111604 1	*Phytophthora colocasiae*	1382	100	97	0 0
	GU111603 1	*Phytophthora citrophthora*	1382	100	97	0 0
	GU111602 1	*Phytophthora citrophthora*	1382	100	97	0 0
	GU111601 1	*Phytophthora citrophthora*	1382	100	97	0 0

Isolates	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e value
	Accession no.	Name				
PSrL-10	JX134651 1	*Phytophthora colocasiae*	1441	100	99	0 0
	JN661144 1	*Phytophthora colocasiae*	1430	100	99	0 0
	AB367509 1	*Phytophthora meadii*	1428	99	99	0 0
	JN618705 1	*Phytophthora botryosa*	1424	98	99	0 0
	KC247914 1	*Phytophthora meadii*	1424	100	99	0 0
	JN661147 1	*Phytophthora colocasiae*	1424	100	99	0 0
	JN661146 1	*Phytophthora colocasiae*	1424	100	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1424	100	99	0 0
	JN661142 1	*Phytophthora colocasiae*	1424	100	99	0 0
	JN661141 1	*Phytophthora colocasiae*	1424	100	99	0 0
PTuL-11	JN661146 1	*Phytophthora colocasiae*	1548	99	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1546	99	99	0 0
	GU111605 1	*Phytophthora colocasiae*	1546	99	99	0 0
	JN661147 1	*Phytophthora colocasiae*	1543	99	99	0 0
	GU111604 1	*Phytophthora colocasiae*	1541	99	99	0 0
	GU111603 1	*Phytophthora citrophthora*	1541	99	99	0 0
	GU111601 1	*Phytophthora citrophthora*	1539	99	99	0 0
	GU133066 1	*Phytophthora citrophthora*	1537	99	99	0 0
	JN661138 1	*Phytophthora colocasiae*	1535	99	99	0 0
	GU111602 1	*Phytophthora citrophthora*	1535	99	99	0 0
PKnL-12	JN661147 1	*Phytophthora colocasiae*	1391	99	97	0 0
	JN661146 1	*Phytophthora colocasiae*	1391	99	97	0 0
	JN661139 1	*Phytophthora colocasiae*	1391	99	97	0 0
	JN661138 1	*Phytophthora colocasiae*	1391	99	97	0 0
	GU111605 1	*Phytophthora colocasiae*	1391	99	97	0 0
	JN661140 1	*Phytophthora colocasiae*	1389	99	97	0 0
	GU111604 1	*Phytophthora colocasiae*	1386	99	97	0 0
	GU111603 1	*Phytophthora citrophthora*	1386	99	97	0 0
	GU111602 1	*Phytophthora citrophthora*	1386	99	97	0 0
	GU111601 1	*Phytophthora citrophthora*	1386	99	97	0 0

Isolates	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e value
	Accession no	Name				
PDeL 13	JN661144 1	*Phytophthora colocasiae*	1424	99	99	0 0
	JN661146 1	*Phytophthora colocasiae*	1421	100	99	0 0
	KC247914 1	*Phytophthora meadii*	1419	99	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1419	99	99	0 0
	JN661142 1	*Phytophthora colocasiae*	1419	99	99	0 0
	JN661141 1	*Phytophthora colocasiae*	1419	99	99	0 0
	GU111605 1	*Phytophthora colocasiae*	1419	99	99	0 0
	GU111604 1	*Phytophthora colocasiae*	1419	99	99	0 0
	JX134651 1	*Phytophthora colocasiae*	1419	99	99	0 0
	KC247922 1	*Phytophthora meadii*	1413	99	99	0 0
PKkL-14	JX134651 1	*Phytophthora colocasiae*	1218	100	95	0 0
	JN661144 1	*Phytophthora colocasiae*	1214	99	95	0 0
	JN661138 1	*Phytophthora colocasiae*	1212	100	94	0 0
	KC247914 1	*Phytophthora meadii*	1208	99	94	0 0
	JN661147 1	*Phytophthora colocasiae*	1208	99	94	0 0
	JN661146 1	*Phytophthora colocasiae*	1208	99	94	0 0
	JN661139 1	*Phytophthora colocasiae*	1208	99	94	0 0
	JN661142 1	*Phytophthora colocasiae*	1208	99	94	0 0
	JN661141 1	*Phytophthora colocasiae*	1208	99	94	0 0
	JN661140 1	*Phytophthora colocasiae*	1208	99	94	0 0
PVaL-15	JX155793 1	*Phytophthora meadii*	1158	99	95	0 0
	AB67509 1	*Phytophthora meadii*	1155	100	94	0 0
	GU259352 1	*Phytophthora meadii*	1153	100	94	0 0
	FJ801908 1	*Phytophthora meadii*	1153	100	94	0 0
	JX134651 1	*Phytophthora colocasia*	1153	100	94	0 0
	JN618738 1	*Phytophthora meadii*	1149	99	94	0 0
	JN618705 1	*Phytophthora meadii*	1149	99	94	0 0
	FJ801306 1	*Phytophthora meadii*	1149	99	94	0 0
	JX134651 1	*Phytophthora colocasia*	1147	100	94	0 0
	JN661144 1	*Phytophthora colocasia*	1147	100	94	0 0

Isolates	NCBI accessions showing maximum homology		Maximum score	Query coverage %	Identity %	e value
	Accession no	Name				
PKuL-16	JN661144 1	*Phytophthora colocasiae*	1469	100	99	0 0
	KC247914 1	*Phytophthora meadii*	1463	100	99	0 0
	JN661147 1	*Phytophthora colocasiae*	1463	100	99	0 0
	JN661146 1	*Phytophthora colocasiae*	1463	100	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1463	100	99	0 0
	JN661142 1	*Phytophthora colocasiae*	1463	100	99	0 0
	JN661141 1	*Phytophthora colocasiae*	1463	100	99	0 0
	JN661140 1	*Phytophthora colocasiae*	1463	100	99	0 0
	GU111605 1	*Phytophthora colocasiae*	1463	100	99	0 0
	GU111605 1	*Phytophthora colocasiae*	1463	100	99	0 0
PPaF-17	JN661144 1	*Phytophthora colocasiae*	1524	99	99	0 0
	JN661147 1	*Phytophthora colocasiae*	1520	100	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1520	100	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1520	100	99	0 0
	JN661140 1	*Phytophthora colocasiae*	1520	100	99	0 0
	GU111605 1	*Phytophthora colocasiae*	1520	100	99	0 0
	JN661142 1	*Phytophthora colocasiae*	1519	99	99	0 0
	GU111604 1	*Phytophthora colocasiae*	1515	100	99	0 0
	GU111603 1	*Phytophthora citrophthora*	1515	100	99	0 0
	GU111602 1	*Phytophthora citrophthora*	1515	100	99	0 0
PMoF-18	JN661147 1	*Phytophthora colocasiae*	1443	100	99	0 0
	JN661146 1	*Phytophthora colocasiae*	1443	100	99	0 0
	JN661139 1	*Phytophthora colocasiae*	1443	100	99	0 0
	JN661140 1	*Phytophthora colocasiae*	1443	100	99	0 0
	JN661138 1	*Phytophthora colocasiae*	1443	100	99	0 0
	GU111605 1	*Phytophthora colocasiae*	1443	100	99	0 0
	JN661145 1	*Phytophthora colocasiae*	1437	100	99	0 0
	GU111604 1	*Phytophthora colocasiae*	1437	100	99	0 0
	GU111603 1	*Phytophthora citrophthora*	1437	100	99	0 0
	GU111602 1	*Phytophthora citrophthora*	1437	100	99	0 0

It is also found that, *Phytophthora* isolates from nutmeg form three major clusters indicating species diversification Of the three major clusters, the first cluster was divided into two sub clusters and isolate PPaL-1 formed a single cluster within the sub cluster The other sub cluster again divided to two, of which one cluster include PPaF-17 and second cluster consisted of PKoL-2 PPoL 3, PMaL 4, PKtL-6, PKaL-7, PThL-9, PSrL 10, PKnL 12, PDeL-13, PKtL-14, PVaL 15,PKuL-16 and PMoF-18 In the second major cluster, two sub clusters were observed consisted of PVeL-5 and PMtL-8 The third major cluster consisted of only one isolate PTuL 11 (Fig 1) Another attempt was done to find out the relationship between 18 isolates and *P ramorum* using ClustalW tool The phylogenic tree showed that isolates PPaL 1, PPaF 17, PMoF 18 and *P ramorum* formed a sub cluster showing very close relationship with each other (Fig 2)

4.7 Host range

Host range study was carried out to find out the infectivity of *Phytophthora* isolates of nutmeg on other known hosts of *Phytophthora* spp *viz* arecanut, coconut, cocoa, rubber, black pepper, cardamom, camboge, vanilla, rose, *Coleopsis*, *Eucalyptus* *Colocasia* and *Citrus* and the symptoms developed on these selected hosts were observed and the description of the symptoms are given below The susceptible hosts showed characteristic symptoms of *Phytophthora* described in that particular host or that of symptoms observed on nutmeg

The results presented in the Table 27 showed that, of the 13 hosts tested only rubber, vanilla, rose, *Coleopsis*, *Eucalyptus* and *Citrus* were found susceptible to *Phytophthora* isolate of nutmeg by producing typical symptom of dark brown water soaked lesions on the midrib of leaves which later spread to leaf lamina and resulted in blighting In cocoa black pepper and *Colocasia* symptom developed as necrotic lesions showing hypersensitive reaction and the crops arecanut, coconut, cardamom and camboge did not show any symptoms (Plate 27) Further conformation with zoospore suspensions of 18 isolates on the detached

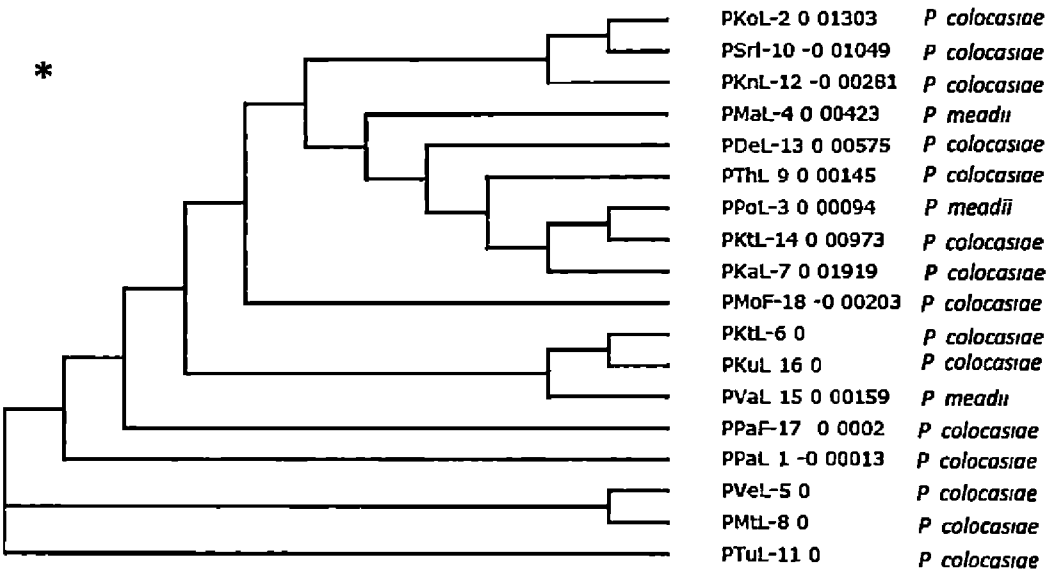


Fig 1. Dendrogram for the 18 *Phytophthora* isolates of nutmeg

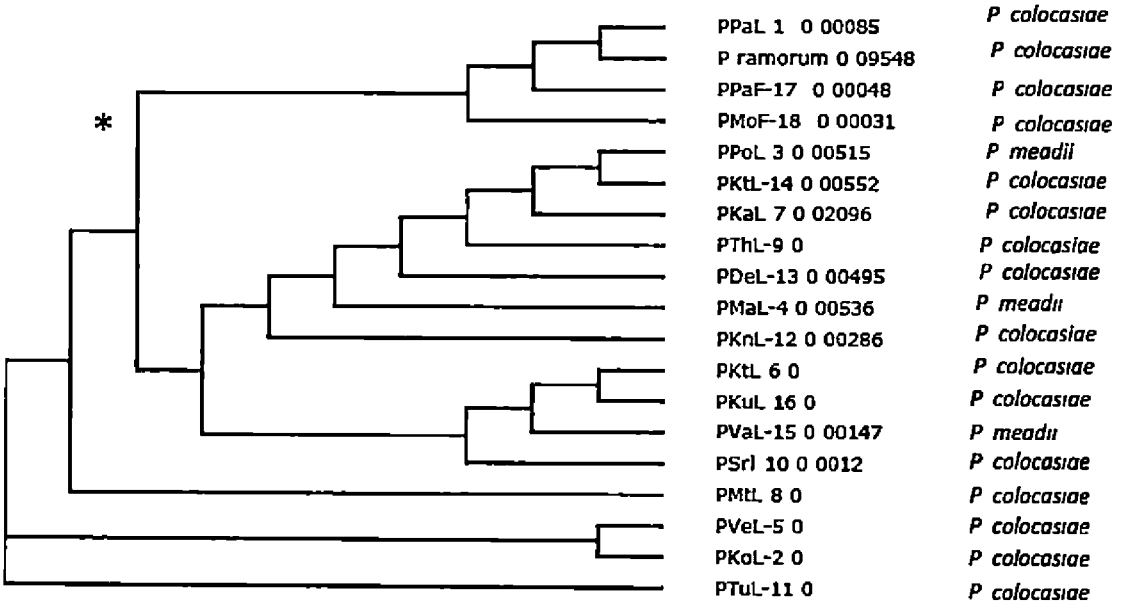


Fig 2 Dendrogram for the 18 *Phytophthora* isolates of nutmeg with *P ramorum*

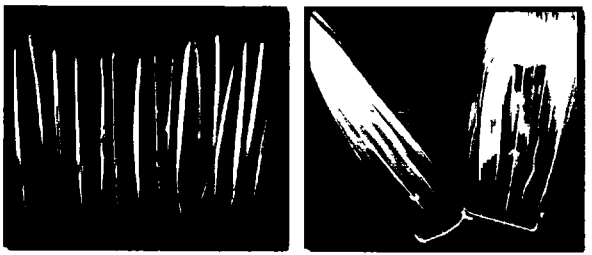
* Names in parenthesis indicate nearest accession on NCBI database

Table 27. Host range of *Phytophthora* isolate of nutmeg

Sl No	Host	Symptom	Days for infection	Days for leaf fall
			Days after inoculation	
1	Nutmeg (control)	Dark brown water soaked lesion on the midrib of the leaves, enlarged & spread to lamina resulted in blightening, premature defoliation Characteristic symptom of *Phytophthora* disease	1	3
2	Arecanut	No symptom	-	-
3	Coconut	No symptom	-	-
4	Cocoa	Small dark brown necrotic lesion with yellow halo Hypersensitive reaction	3	No leaf fall
5	Rubber	Dark brown water soaked lesion on the midrib of the leaves, enlarged & spread to lamina resulted in blightening, coagulated latex on the midrib, bronzing of the young foliage, premature defoliation – Characteristic symptom of *P meadii* on rubber	1	3
6	Black pepper	Hypersensitive reaction	3	No leaf fall
7	Cardamom	No symptom		-
8	Camboge	No symptom	-	-
9	Vanilla	Characteristic symptoms of *P meadii* on vanilla	2	-
10	Rose	Characteristic symptoms of *P ramorum* on rose	1	3
11	*Coreopsis*	Characteristic symptoms of *Phytophthora* disease of nutmeg	2	
12	*Colocasia*	Hypersensitive reaction	4	No leaf fall
13	*Eucalyptus*	Characteristic symptoms of *Phytophthora* on *Eucalyptus*	2	No leaf fall
14	*Citrus*	Characteristic symptoms of *P citrophthora* on *Citrus*	2	4

Plate 27. Host range of *Phytophthora* of nutmeg

A. Symptom on coconut



B. Symptom on arecanut

C. Symptom on cardamom

D. Symptom on camboge

leaves of these host under lab condition also yielded same result and all isolates showed same type of symptom on that particular susceptible host

The pathogen could be reisolated from the hosts which developed water soaked lesion and showed typical characters of the original culture and the pathogenicity could be proved However, the reisolation of the pathogen from the hosts which showed hypersensitive reaction, failed to produce any fungal growth on the media

Hence, it is concluded that, rubber, vanilla rose *Coreopsis*, *Eucalyptus* and *Citrus* are the hosts of *Phytophthora* isolate of nutmeg

4.8 1 Symptoms on rubber

Initial symptom was developed after first day of inoculation with culture disc whereas it was 10 days for zoospore suspension Symptom first appeared as light brown discoloration on the midrib which later turned to dark brown water soaked lesion and spread along the lateral veins to leaf lamina Coagulated latex was also observed on the midrib Another characteristic symptom was the bronzing of the young foliage Petioles of the infected leaves showed black discoloration resulted in premature defoliation of leaves in three days after inoculation All the 18 isolates showed same type of symptoms (Plate 28 A)

4 8 2 Symptoms on vanilla

Vanilla leaves inoculated with all the 18 *Phytophthora* isolates produced same symptom Initial symptom was observed at 2 and 11 DAI by culture disc and zoospore inoculation methods respectively The symptom appeared as black water soaked lesion which enlarged and spread to entire leaf lamina resulted in rotting of the leaves (Plate 28 B)

4 8.3 Symptoms on *Eucalyptus*

Initial symptom developed 2 and 11 DAI by culture disc and zoospore inoculation methods respectively The symptom first appeared as black water

soaked lesions on the midrib of leaves, which enlarged and spread along the vein and veinlet to entire lamina and no defoliation was noticed (Plate 28 C)

4.8 4 Symptoms on rose

Initial symptom was noticed at 1st day after inoculation with culture disc method Symptom appeared as dark brown water soaked lesion at the midrib which spread along the lateral veins and leaves turned yellow and defoliated at 3 DAI

With zoospore inoculation, symptom appeared 5 DAI as water soaked lesion on the leaves and petiole Both green and yellow leaves defoliated at 7 DAI Water soaked lesion was also observed on buds led to rotting of buds, which spread to flower stalk and resulted in shedding of buds (Plate 29 A)

4 8 5 Symptoms on *Coreopsis*

On inoculation with the 18 isolates on detached leaves, the symptom was observed only with isolate PPaL-1, PDeL-13 and PMoF 18 Infection started at two days after inoculation as black water soaked lesion on the inoculated area, which later spread along the vein and veinlets to entire lamina causing rotting of the leaves (Plate 29 B)

4 8.6 Symptoms on *Citrus*

Citrus leaves inoculated with the 18 *Phytophthora* isolates produced typical water soaked symptom at 2 DAI The symptom first appeared a slight brown water soaked lesion on the midrib of leaves which enlarged and spread along the vein and veinlet and resulted in degreening of the leaves Infected leaves defoliated on 4 DAI (Plate 29 C)

4.8.7 Symptoms on cocoa, black pepper and *Colocasia*

Inoculation of virulent *Phytophthora* isolate showed initial infection at 3 DAI in cocoa and black pepper and 4 days in *Colocasia* The infection appeared as small black lesion with yellow halo, which later became necrotic without further spread, showing hypersensitive reaction However, no symptom was observed on

Plate 28. Host range of *Phytophthora* of nutmeg

A. Symptom on rubber

Symptom on detached leaves

Symptom on seedling

B. Symptom on vanilla

3 days after inoculation

7 days after inoculation

C. Symptom on *Eucalyptus*

Symptom on detached leaves

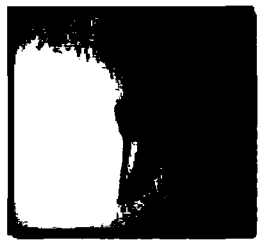
Symptom on seedling

Plate 29. Host range of *Phytophthora* of nutmeg

A. Symptom on rose

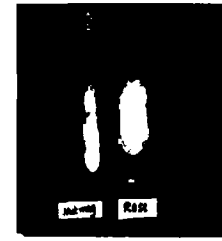
Symptom on detached leaves

Symptom on plant



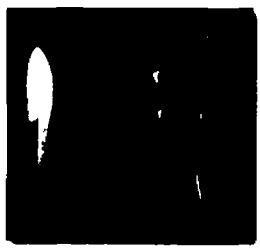
Symptom on bud

Defoliation



Reisolation of pathogen

B. Symptom on *Coreopsis*



Symptom on detached leaves

Symptom under natural condition

C. Symptom on *Citrus*

Symptom on detached leaves

detached leaves of cocoa, black pepper and *Colocasia*, on inoculation with the zoospore suspension of the 18 *Phytophthora* isolates under lab condition (Plate 30)

4 9 Cross infectivity studies

In order to study the infectivity of other *Phytophthora* spp on nutmeg, various *Phytophthora* spp *viz P palmivora* of coconut and cocoa, *P meadii* of arecanut, rubber, cardamom and vanilla *P capsici* of black pepper, *P colocasiae* of *Colocasia* and *P citrophthora* of *Citrus* were inoculated on the leaves of nutmeg seedlings and the observations were presented in Table 28

Among the different species of *Phytophthora* tested, only *P meadii* of vanilla and *P citrophthora* of *Citrus* produced typical water soaked lesion on nutmeg leaves Symptom appeared as dark brown water soaked lesion on the midrib of the leaves at 2 DAI, which enlarged and spread along the lateral veins to leaf lamina resulted in blighting which is the typical symptoms that observed with the nutmeg leaf fall disease, indicating the positive result of cross infectivity of *P meadii* of vanilla and *P citrophthora* of *Citrus* on nutmeg It is also noted that, arecanut and cardamom isolates of *P meadii* developed necrotic spots at 4 DAI, however no symptom could be observed with the isolate of rubber, eventhough it was a collateral host of nutmeg *Phytophthora* Likewise, *P palmivora* of coconut and cocoa, *P capsici* of black pepper and *P colocasiae* of *Colocasia* also showed hypersensitive reaction indicating non hosts of the pathogen (Plate 31)

Thus the study indicated that, nutmeg is a collateral host of *P meadii* of vanilla and *P citrophthora* of *Citrus* and non hosts of *P palmivora*, *P capsici* *P colocasiae* and *P meadii* of arecanut, rubber and cardamom

On inoculation of different *Phytophthora* spp on rose, rubber, arecanut, cardamom and vanilla isolates of *P meadii* and *P citrophthora* of *Citrus* showed typical symptoms at 2 DAI on rose (indicating that rose is also a host of *P meadii*) whereas *P palmivora* of coconut and cocoa, *P capsici* of black pepper

Plate 30. Host range of *Phytophthora* of nutmeg

A. Symptom on pepper

Symptom on detached leaves

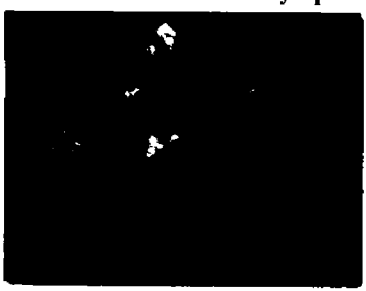
Symptom on seedlings

B. Symptom on cocoa

Symptom on detached leaves

Symptom on seedlings

C. Symptom on *Colocasia*



Symptom on detached leaves

Symptom on plant

Table 28 Cross infectivity of various *Phytophthora* spp on nutmeg

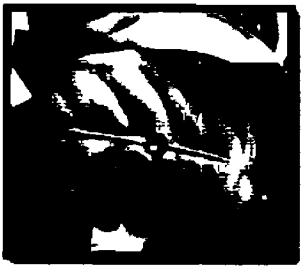
Pathogen	Host	Symptoms on nutmeg leaves	Initial infection (DAI)	Lesion size (cm)
P palmivora	Coconut	Black necrotic spot on moculated area – Hypersensitive reaction	5	0 1-1
P palmivora	Cocoa	Small dark brown necrotic spot on inoculated area – Hypersensitive reaction	4	0 1-0 5
P meadii	Arecanut	Small black necrotic spot with yellow halo – Hypersensitive reaction	4	0 5-1
P meadii	Rubber	No symptom	0	0
P meadii	Cardamom	Small dark brown necrotic spot on inoculated area – Hypersensitive reaction	4	0 1-0 5
P meadii	Vanilla	Dark brown water soaked lesion on the midrib of the leaves, spread to leaf lamina resulted in blighting – characteristic symptom of *Phytophthora* on nutmeg	2	Full leaf
P capsici	Black pepper	Small brown necrotic spot with yellow halo – Hyper sensitive reaction	5	0 5-1
P colocasiae	*Colocasia*	Small dark brown necrotic spot on inoculated area – Hyper sensitive reaction	5	0 1-0 5
P citrophthora	*Citrus*	Dark brown water soaked lesion on the midrib of the leaves, spread to leaf lamina resulted in blighting - characteristic symptom of *Phytophthora* on nutmeg	2	Full leaf

DAI – Days after inoculation

Plate 31.

Cross infectivity of various *Phytophthora* spp. on nutmeg

P. palmivora of coconut



P. palmivora of cocoa

P. capsici of black pepper

P. meadii of rubber

P. meadii of arecanut

P. meadii of vanilla

P. colocasiae of *Colocasia*

P citrophthora of citrus

and *P colocasiae* of *Colocasia* failed to take infection on rose Similarly, *P citrophthora* also showed infectivity on *Eucalyptus* and *Coreopsis* and the pathogen could be reisolated Eventhough *P meadii* of vanilla showed typical symptom on *Eucalyptus*, reisolation of the pathogen was failed to confirm its pathogenicity on this host

From the data presented in Table 29, it is evident that, eventhough the 15 *Phytophthora* isolates of nutmeg showed 95-99 per cent molecular similarity with *P colocasiae,* they varied in cultural and most of the morphological characters except in caducous nature of sporangia and chlamydospore characters It is also noted that, none of the *Phytophthora* isolates of nutmeg could cause infection on *Colocasia* and likewise, *Phytophthora* of *Colocasia* could cause infection on nutmeg Similarly, three isolates of nutmeg showed 94-99 per cent molecular similarity and also in cultural and sporangial dimensions especially the pedicel length with *P meadii* The main dissimilarity was observed with respect to chlamydospore production, in which it was rare in *P meadii* but was abundant in *Phytophthora* isolates of nutmeg It is also noted that, this pathogen could develop symptoms only on rubber and vanilla and failed to cause infection on arecanut and cardamom, the other known hosts of *P meadii* Among the different isolates of *P meadii* tested, only vanilla isolate showed infection on nutmeg Eventhough *Phytophthora* isolates of nutmeg showed similarity to *P citrophthora* in molecular characters, it differed in both cultural and morphological character However, showed positive results in host range and cross infectivity studies *Phytophthora* isolates of nutmeg showed very much similarity to cultural and many of the morphological characters of *P ramoram* but showed only 89 per cent molecular similarity Moreover, some of the reported hosts (Rose, *Eucalyptus* and *Citrus*) of *P ramoram* was found to be the host of *Phytophthora* isolates of nutmeg

Table 29 Comparision of *Phytophthora* isolates of nutmeg with *Phytophthora* sp. showing molecular similarity

Characters	*P. colocasiae*	*P citrophthora*	*P meadii*	*P ramorum*
Cultural characters	-		+	+
Sporangial characters				
• Caducous	+	-	+	+
• Papilla	+	-	-	+
• Shape			-	+
• Length	-	-	+	+/
• Breadth	-	-	+	+/-
• L/B ratio	-		+	+/
• Pedicel length	-	-	+	-
Chlamydospore				
• Adundant	+	-	Rare	+
• Shape	+	+	+	+
• Size	+	-	-	-
Molecular characterisation	95 99%	97-99%	94-99%	89%
Host range	-	+ (*Citrus*, Rose, Rubber)	+ (Rubber, Vanilla)	+ (Rose, *Eucalyptus*, *Citrus*)
Cross infectivity		+	+ (*P meadii* of vanilla)	

4.10 Identification of *Trichoderma sp* isolated from nutmeg

Trichoderma isolates TN-1 and TN-3 obtained from nutmeg rhizosphere soil were identified as *T viride* [ID No NCFT- 5638 67 (TN-1) and ID No NCFT 5639 67 (TN-3)] and TN-2 as *T harzianum* [ID No NCFT- 5635 67 (TN-2)] by National Centre for Fungal Taxonomy (NCFT), New Delhi

4.11 Disease management

Eight fungicides and six bioagents were screened for their inhibitory effect against the most virulent *Phytophthora* isolate (PPaL 1) of nutmeg under *in vitro* and *in vivo* condition

4.11 1 *In vitro* evaluation of fungicides

The inhibitory effect of different fungicides on *Phytophthora* was studied by poisoned food technique The results of the experiment is furnished in Table 30

From the data presented in Table, it is observed that, all chemicals showed more than 90 per cent inhibition of the pathogen except the combination product of carbendazim + mancozeb 1% Bordeaux mixture, potassium phosphonate (3 ml/l) and the plant activator (3 ml/l) and the combination fungicide iprovalicarb + propineb at lower (1 5 g/l) and higher (2 g/l) concentrations showed cent per cent inhibition of pathogen Lower doses of copper hydroxide (1 5 g/l), copper oxychloride (2 g/l) and cymoxanil + mancozeb (1 5g/l) showed about 90 per cent inhibition, however complete inhibition of the pathogen was observed with the higher doses of 2 0, 2 5 and 2 0 g/l of these fungicides respectively The combination fungicide carbendazim +mancozeb, was found to be least effective recording only 70 55 per cent inhibition (Plate 32 A)

Based on *in vitro* screening, eight fungicides each at one most effective concentration, *viz* Bordeaux mixture (1%), copper hydroxide (2g/l), copper oxychloride (2 5g/l), cymoxanil + mancozeb (2g/l), iprovalicarb + propineb

Table 30 ***In vitro*** **evaluation of selected fungicides against** ***Phytophthora*** **isolate of nutmeg**

Sl. No	Chemical name	Concentration	*Mean colony diameter (cm)	Per cent inhibition
1	Bordeaux Mixture	1%	0	100
2	Copper hydroxide77WP	1 5g/l	0 85	90 5
		2g/l	0	100
3	Copper oxychloride 50 WP	2g/l	0 86	90 4
		2 5g/l	0	100
4	Cymoxaml 8% + mancozeb 64% WP	1 5g/l	0 82	90 9
		2g/l	0	100
5	Iprovalicarb 5 5% + propineb 61 3% WP	I 5g/l	0	100
		2g/l	0	100
6	Carbendazim 12%+mancozeb 63% WP	2g/l	2 65	70 55
7	Potassium phosphonate 50%	3ml/l	0	100
8	Potassium phosphonate + Phytoalexin	3ml/l	0	100

* Mean of three replications

(1 5g/l), carbendazim +mancozeb (2g/l) potassium phosphonate (3ml/l) and plant activator (3ml/l) were selected for *in vivo* experiment

4 11.2 *In vitro* screening of antagonists against the pathogen

Trichoderma viride -1, *T harzianum* and *T viride* -2 the isolates from nutmeg and the reference cultures *viz T viride*, *T harzianum* and *Pseudomonas fluorescens* were screened against *Phytophthora* isolate of nutmeg and the findings are presented in Table 31

It was evident from the data that, all the six antagonists showed antagonistic activity against the pathogen and all *Trichoderma* sp showed cent per cent inhibition at 4 DAI by the overgrowth mechanism of antagomsm, causing complete disintegration of the pathogen However, the bacterial antagonist, *P fluorescens*, showed only 61 11 per cent inhibition (Plate 32B) Of the five *Trichoderma* isolates, *T viride* of KAU and *T viride* -1 of nutmeg showed faster inhibition and growth with mean colony diameter of 6 8 and 6 3 cm respectively at 3 DAI and these two isolates were selected for *in vivo* experiment

4 11 3 *In vivo* experiment for disease management

An *in vivo* experiment was carried out to evaluate the efficacy of the selected fungicides and antagomsts in the management of *Phytophthora* causing leaf fall disease of nutmeg Eight fungicides and two antagonists which showed higher efficiency in *in vitro* screening were selected for *in vivo* experiment Observations on disease incidence and severity were recorded and the results are summarized in Table 32 and 33

4.11.3.1 Effect of treatments on per cent disease incidence

Observations on disease incidence recorded at 10, 15 and 20 days after inoculation is summarized in Table 32 As artificial inoculation was given, infection was noticed in almost all the inoculated leaves recording 86 to 100 per cent incidence in chemical treatments, whereas in case of bioagents, the incidence

Table 31 *In vitro* screening of antagonists against pathogen

Antagonists	*Mean colony diameter (cm)										Per cent inhibition of the pathogen
	Days after incubation										
	1		2		3		4		5		
	A	P	A	P	A	P	A	P	A	P	
Trichoderma viride 1	1 3	2 1	3 8	3 4	6 3	2 7	9	-	9	-	100
T harzianum	1 0	1 8	3 5	3 1	6 1	2 9	9		9		100
T viride -2	1 3	1 7	3 7	3 3	6 1	2 9	9	-	9	-	100
T viride (Reference culture KAU)	1 4	2 0	3 8	3 2	6 8	2 2	9		9	-	100
T harzianum (Reference culture IISR)	1 1	1 7	3 6	2 8	6 2	2 8	9	-	9	-	100
Pseudomonas fluorescens (Reference culture KAU)	-	1 0	-	2 4		3 5		3 5	-	3 5	61 11
Control (Pathogen alone)		2 5	-	4 3	-	6 4	-	8 1	-	9 0	-

* Mean of three replications

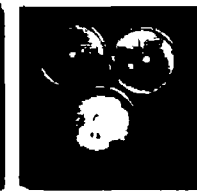
Plate 32. *In vitro* evaluation of fungicides and antagonists

A. *In vitro* screening of selected fungicides against *Phytophthora*

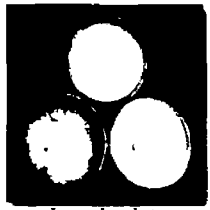
Bordeaux Mixture (1%)

Copper hydroxide (2g/l)

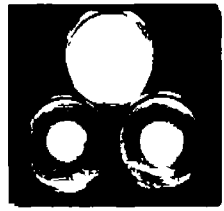
Copper oxychloride (2 5g/l)



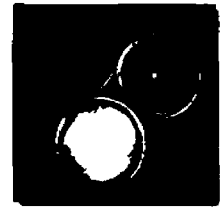
Cymoxani +mancozeb (2g/l)



Iprovalicarb + propineb (1 5g/l)



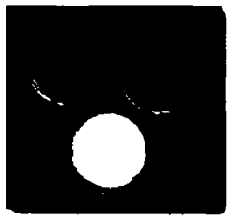
Carbendazim + mancozeb (2g/l)



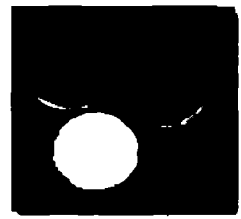
Potassium phosphonate (3ml/l)

Plant activator (3ml/l)

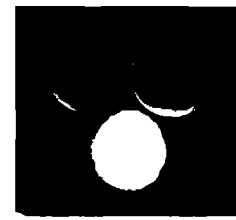
B. *In vitro* evaluation of antagonists against *Phytophthora*



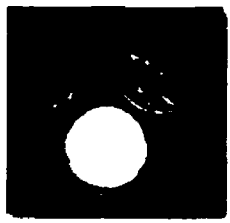
T viride 1 (Nutmeg)



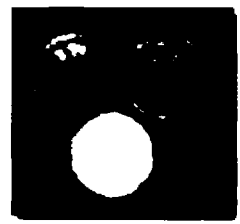
T harzianum (Nutmeg)



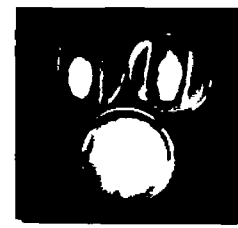
T viride 2 (Nutmeg)



T viride (KAU)



T harzianum (IISR)



P fluorescens (KAU)

Table 32. Effect of treatments on per cent disease incidence

Treatment No	Treatments	Per cent disease incidence		
		Days after inoculation		
		10	15	20
T_1	1% Bordeaux Mixture spray	88 00 (9 38)^{def}	88 00 (9 38)^{de}	88 67 (9 42)^{de}
T_2	Copper oxychloride (2 5g/l) spray	87 33 (9 35)^{ef}	89 33 (9 45)^{cde}	89 33 (9 45)^{cde}
T_3	Copper hydroxide (2g/l) spray	85 33 (9 24)^{f}	85 33 (9 24)^{e}	86 00 (9 27)^{e}
T_4	Cymoxanil + mancozeb (2g/l) spray	90 67 (9 52)^{cdef}	90 67 (9 52)^{bcde}	90 67 (9 52)^{bcde}
T_5	Iprovalicarb +propineb (1 5g/l) spray	96 00 (9 80)^{abc}	96 00 (9 80)^{abc}	97 33 (9 87)^{ab}
T_6	Potassium phosphonate (3ml/l) spray	97 33 (9 87)^{ab}	97 33 (9 87)^{ab}	98 67 (9 93)^{a}
T_7	Carbendazim +mancozeb 63 (2g/l) spray	88 66 (9 42)^{def}	88 66 (9 42)^{de}	89 33 (9 45)^{cde}
T_8	Plant activator (3ml/l) spray	88 66 (9 41)^{def}	88 66 (9 41)^{de}	88 66 (9 41)^{de}
T_9	Copper oxychloride (2 5g/l) soil drenching	92 67 (9 63)^{abcde}	92 67 (9 63)^{abcd}	94 67 (9 73)^{abcd}
T_{10}	Copper hydroxide (2g/l) soil drenching	89 33 (9 45)^{def}	89 33 (9 45)^{cde}	90 67 (9 52)^{bcde}
T_{11}	Cymoxanil + mancozeb (2g/l) soil drenching	88 00 (9 38)^{def}	88 00 (9 38)^{de}	88 67 (9 42)^{de}

Treatment No	Treatments	Per cent disease incidence		
		Days after inoculation		
		10	15	20
T_{12}	Iprovalicarb +propineb (2g/l) soil drenching	89 33 (9 45)^{def}	89 33 (9 45)^{cde}	89 33 (9 45)^{cde}
T_{13}	1% Bordeaux mixture spray + copper oxychloride (2 5g/l) soil drenching	91 33 (9 56)^{bcdef}	91 33 (9 56)^{bcde}	91 33 (9 56)^{bcde}
T_{14}	1% Bordeaux mixture spray + copper hydroxide (2g/l) soil drenching	96 00 (9 80)^{abc}	97 33 (9 87)^{ab}	97 33 (9 87)^{ab}
T_{15}	Copper oxychloride (2 5g/l) spray and soil drenching	98 00 (9 90)^{abcd}	98 66 (9 93)^{abcd}	98 66 (9 93)^{abcd}
T_{16}	Copper hydroxide (2g/l) spray and soil drenching	90 66 (9 52)^{cdef}	90 66 (9 52)^{bcde}	92 00 (9 59)^{abcd}
T_{17}	1% Bordeaux mixture spray and *T viride* (KAU)soil application	90 00 (9 48)^{cdef}	90 00 (9 48)^{cde}	90 00 (9 48)^{cde}
T_{18}	2% *P fluorescens* spray and *T viride* (KAU) soil application	40 00 (6 33)^{h}	41 33 (6 41)^{b}	41 33 (6 41)^{b}
T_{19}	*T viride* of nutmeg – soil application	46 66 (6 82)^{g}	46 66 (6 82)^{f}	46 66 (6 82)^{f}
T_{20}	*T viride* (KAU) – soil application	46 66 (6 82)^{b}	46 66 (6 82)^{f}	46 66 (6 82)^{f}
T_{21}	Control (inoculation of pathogen on leaves)	100 00 (10 0)^{a}	100 00 (10 0)^{a}	100 00 (10 0)^{a}
T_{22}	Control (soil application of pathogen)	92 00 (9 59)^{abcd}	92 00 (9 59)^{abcd}	96 00 (9 80)^{abc}
CD(0 05)		0 33	0 35	0 36

*Mean of three replications
Figures in parenthesis are transformed values

was less as treatments were given prophylactic and showed only 41 33 46 66 against 96-100 per cent in control

From the data, it is found that, per cent disease incidence was more than 85 per cent in all treatments at all the three intervals of observations, except antagonists application, of which, T_{18} (Prophylactic spraymg 2% *P fluorescens* and soil application of *T viride* of KAU) recorded lowest incidence (40 per cent) against 96 - 100 per cent in control which was followed by the treatment T_{19} (soil application of *T viride* of nutmeg) and T_{20} (soil application of *T viride* - KAU) with 46 66 per cent at 10 DAI The same trend was noticed at 15 and 20 DAI Among the various treatments, maximum disease incidence of (98 66 per cent) was noticed in T_{15} (spraying and soil drenching of copper oxychloride - 2 5g/l) All other treatments belonged to a homogenous subgroup and not much variation was noticed in incidence at different intervals indicating the non - spread of the disease to unmoculated plant parts

4.11 3.1 Effect of treatments on per cent disease severity

Per cent disease severity of leaf fall disease of nutmeg was recorded for each treatment at 10, 15 and 20 DAI and results are furnished in Table 33 It is observed from the data that, all treatments were superior to control and significant difference was noticed among the treatments at all intervals of observations (Plate 33) Among the treatments, spraying of 1% Bordeaux mixture + soil drenching of copper hydroxide- 2g/l (T_{14}) showed lowest disease severity at all intervals of observations recording 17 87, 17 87 and 19 2 per cent followed by spraying of 1% Bordeaux mixture + soil application of *T viride* - KAU (T_{17}) with 18 23, 18 67 and 19 73 per cent against 100 and 43 33, 100 and 66 67 and 100 and 80 67 in control (T_{21} and T_{22}) at 10, 15 and 20 DAI respectively and showed 79 43 and 78 86 per cent reduction over control In addition, spraying of 1% Bordeaux mixture + soil drenching of copper oxychloride, spraying and drenching of copper hydroxide or copper oxychloride and even spraying of Bordeaux mixture alone were found equally effective, recording only 20 0 - 20 6 per cent severity, with

Table 33 Effect of treatments on per cent severity of *Phytophthora* leaf fall of nutmeg

Treatment. No	Treatments	*Per cent disease severity Days after inoculation			Per cent reduction over control	Progress of infection (%)	Per cent leaf fall
		10	15	20		10 – 20 DAI	10- 20 DAI
T_1	1% Bordeaux Mixture spray	19 73 $(4\ 44)^{efg}$	20 00 $(4\ 47)^{hykl}$	20 60 $(4\ 54)^{ghij}$	77 93	0 87	1 33
T_2	Copper oxychloride (2 5g/l) spray	20 80 $(4\ 56)^{cdefg}$	22 67 $(4\ 76)^{defgh}$	23 20 $(4\ 82)^{def}$	75 14	2 40	2 00
T_3	Copper hydroxide (2g/l) spray	20 67 $(4\ 55)^{cdefg}$	22 13 $(4\ 70)^{efghi}$	22 93 $(4\ 79)^{ef}$	75 43	2 26	1 33
T_4	Cymoxanil + mancozeb (2g/l) spray	20 00 $(4\ 47)^{efg}$	20 80 $(4\ 56)^{fghijk}$	21 87 $(4\ 67)^{fghi}$	76 57	1 87	
T_5	Iprovalicarb +propineb (1 5g/l) spray	20 00 $(4\ 47)^{efg}$	20 67 $(4\ 55)^{fghijk}$	21 33 $(4\ 62)^{fghij}$	77 15	1 33	-
T_6	Potassium phosphonate (3ml/l) spray	22 67 $(4\ 76)^{cde}$	23 33 $(4\ 83)^{def}$	24 53 $(4\ 95)^{de}$	73 72	1 86	2 00
T_7	Carbendazim +mancozeb (2g/l) spray	20 67 $(4\ 76)^{cde}$	23 33 $(4\ 83)^{def}$	23 47 $(4\ 84)^{def}$	74 85	2 8	2 66
T_8	Plant activator (3ml/l) spray	20 53 $(4\ 53)^{defg}$	21 87 $(4\ 67)^{efghi}$	22 4 $(4\ 73)^{efghi}$	76 00	1 87	2 00
T_9	Copper oxychloride(2 5g/l) soil drenching	24 00 $(4\ 89)^{c}$	26 67 $(5\ 16)^{c}$	27 33 $(5\ 23)^{c}$	66 12	3 33	2 00
T_{10}	Copper hydroxide (2g/l) soil drenching	22 67 $(4\ 76)^{cde}$	25 33 $(5\ 03)^{cd}$	25 33 $(5\ 032)^{cd}$	68 61	2 66	1 33
T_{11}	Cymoxanil + mancozeb (2g/l) soil drenching	21 60 $(4\ 65)^{cdef}$	22 67 $(4\ 76)^{defgh}$	23 33 $(4\ 83)^{def}$	71 08	1 73	-
T_{12}	Iprovalicarb +propineb (2g/l) soil drenching	20 00 $(4\ 47)^{efg}$	20 27 $(4\ 450^{ghijkl}$	21 20 $(4\ 60)^{fghij}$	73 72	1 2	-
T_{13}	1% Bordeaux mixture spray + copper oxychloride (2 5g/l) soil drenching	18 67 $(4\ 32)^{fg}$	19 20 $(4\ 38)^{defgh}$	20 26 $(4\ 501)^{hij}$	78 29	1 59	1 33

Treatment .No	Treatments	*Per cent disease severity			Per cent reduction over control	Progress of infection (%)	Per cent leaf fall
		Days after inoculation					
		10	15	20		10 – 20 DAI	10- 20 DAI
T_{14}	1% Bordeaux mixture spray+ copper hydroxide (2g/l) soil drenching	17 87 (4 23)^{g}	17 87 (4 23)^{l}	19 20 (4 38)^{j}	79 43	1 33	-
T_{15}	Copper oxychloride (2 5g/l) spray and soil drenching	18 67 (4 32)^{fg}	19 47 (4 41)^{ijkl}	20 53 (4 53)^{ghij}	78 00	1 86	-
T_{16}	Copper hydroxide (2g/l) spray and soil drenching	18 67 (4 32)^{fg}	19 20 (4 38)^{jkl}	20 00 (4 47)^{ij}	78 57	1 33	-
T_{17}	1% Bordeaux mixture spray and T *viride* (KAU)soil application	18 23 (4 27)^{g}	18 67 (4 32)^{kl}	19 73 (4 44)^{ij}	78 86	1 5	1 33
T_{18}	2% *P fluorescens* spray and *T viride* (KAU) soil application	20 27 (4 50)^{defg}	21 6 (4 65)^{efghij}	22 67 (4 76)^{efg}	71 90	2 4	-
T_{19}	*T viride* of nutmeg soil application	23 33 (4 83)^{cd}	24 00 (4 90)^{cde}	24 67 (4 97)^{de}	69 42	1 34	-
T_{20}	*T viride* (KAU) soil application	21 60 (4 65)^{cdef}	22 93 (4 79)^{defg}	24 33 (5 03)^{cd}	68 60	2 73	-
T_{21}	Control (inoculation of pathogen on leaves)	100 (10 0)^{a}	100 (10 0)^{a}	100 (10 0)^{a}	-	Full infection	100
T_{22}	Control (soil application of pathogen)	43 33 (6 58)^{b}	66 67 (8 162)^{b}	80 67 (8 98)^{b}	-	37 33	-
CD (0 05)		0 343	0 290	0 243	-	-	

*Mean of three replications
DAI- Days after inoculation
Figures in parenthesis are transformed values

Plate 33. Disease management under *in vivo* condition

T_1- 1% Bordeaux mixture spray

T_{12}- Iprovalicarb + propineb soil drenching

T_{14}- 1% Bordeaux mixture spray + copper hydroxide drenching

T_{17}- 1% Bordeaux mixture spray + *T viride* soil application

T_{18}- *P fluorescens* spray + *T viride* soil application

T_{19}- *T viride* (Nutmeg) soil application

T_{20}- *T viride* (KAU) soil application

T_{21}- Control (Leaf inoculation)

T_{22}- Control (Sod application)

78 6 – 77 9 per cent disease reduction at 20 DAI Among the treatments, minimum disease reduction (66 1 per cent) was observed in soil drenching of copper oxychloride alone

It is also noted that, statistical analysis of the data on disease severity at 15 and 20 DAI revealed no significant difference from the observations at 10 DAI except in control (T_{21} and T_{22}) which indicate the positive effect of treatments on the spread of infection

Table 33 also indicated that, per cent severity varied significantly with method of application of fungicide and antagonists Among fungicidal spraying alone,minimum severity (20 6 per cent) was recorded in spraying of 1% Bordeaux mixture followed iprovalicarb +propineb1 5g/l (21 33 per cent) and cymoxanil + mancozeb 2g/l (21 87 per cent) against cent per cent in control at 20 DAI

In soil drenching method, lowest severity (21 2 per cent) was noticed in drenching of iprovalicarb + propineb -2g/l (T_{12}) which was on par with cymoxanil + mancozeb 2g/l (23 33 per cent) and copper hydroxide 2g/l (25 33 per cent) while the maximum severity (27 33 per cent) was in copper oxychloride - 2 5g/l (T_9) It is observed that, soil drenching with fungicides containing systemic chemicals showed superior effect than the contact fungicides alone

Application of chemical by both spraying and drenching showed minimum severity (19 47 per cent) with 1% Bordeaux mixture spray + copper hydroxide drenching and the other three treatments, consisted of 1% Bordeaux mixture + copper oxychloride (T_{13}), spraying and drenching of copper hydroxide (T_{16}) / copper oxychloride (T_{15}) were statistically on par

Among the bioagents, prophylactic spraying 2% *P fluorescens* and soil application of *T viride* of KAU (T_{18}) recorded the minimum severity of 20 27 per cent and was on par with soil application of *T viride* of nutmeg (T_{19}) / *T viride* of KAU (T_{20})

It is also observed from the data that, disease progress was very less in all treatments ranged from 0 87 – 3 33 per cent against 37 33 per cent and full infection in control, at different intervals of observation Analysis of data on per cent disease progress revealed that, the progress of infection was minimum (0 87 per cent) in seedlings treated with 1% Bordeaux mixture spray (T_1) followed by application of iprovalicarb +propineb as soil drench (1 2 per cent) or spray (1 33 per cent) The maximum progress was noticed in T_9 (soil drenching of copper oxychloride 2 5g/l) with 3 33 per cent Control treatments recorded full infection and 37 33 per cent for inoculation of pathogen on leaves and soil respectively

Similarly, while analyzing the per cent leaf fall in different treatments, it is noted that, the leaf fall symptom was noticed only with the inoculation of the pathogen on the foliage However, no leaf fall symptom was observed in T_4 (spraying of cymoxaml + mancozeb), T_5 (spraying of iprovalicarb +propineb), T_{14} (1% Bordeaux mixture spray + soil drenching of copper hydroxide), T_{15} (spraying and soil drenching of copper oxychloride) and T_{16} (spraying and soil drenching of copper hydroxide) and the leaf fall per cent was very less in other treatments also, which ranged only from 1 33 to 2 66 per cent against cent per cent in control Application of inoculum to the soil showed only die back symptom

Summing up the findings of *in vivo* study, it is observed that, the treatments recorded 79 4 - 66 1 per cent reduction of disease of which, spraying of 1% Bordeaux mixture + soil drenching of copper hydroxide and spraying of 1% Bordeaux mixture and soil application of *T viride* showed maximum reduction of the disease It is also noticed that, the treatments consisted of Bordeaux mixture spray were more effective as compared to other fungicidal treatments and spraying of 1% Bordeaux mixture alone was also superior to other treatments Thus, these results also showed that, all copper fungicides tested under study were effective against *Phytophthora* of nutmeg of which, Bordeaux mixture was the most effective one followed by copper hydroxide and copper oxychloride Likewise, soil drenching of systemic fungicide iprovalicarb + propineb (T_{12}) was found to be more effective among the soil treatments and was on par with drenching of cymoxanil +

mancozeb (T_{11}) and copper hydroxide (T_{10}) However, both spraying + soil application of fungicides/ bioagents showed better result as compared to spraying or drenching alone It is also noted that, among the biocontrol treatments, prophylactic spraying of 2% *P fluorescens* + soil application of *T viride* was most effective control and was on par with soil application of *Trichoderma* alone

Discussion

5. DISCUSSION

Nutmeg, an evergreen tree spice seen in tropics is grown for its kernel and mace Nutmeg is popular for its flavouring and therapeutic properties and is the one of the costliest spices in global market In India, Kerala forms the major area of nutmeg cultivation Ernakulam, Thrissur, Idukki and Kottayam are the major nutmeg producing districts in the state Recently, nutmeg farmers in these districts are facing severe crop loss due to massive attack of *Phytophthora* sp causing heavy leaf fall during South – West monsoon period In this view, an investigation was carried out to study about the disease and the pathogen associated with it, for the better management strategies

Since the leaf fall disease is a serious problem in major nutmeg growing areas of Ernakulam, Thrissur and Kottayam districts of Kerala, the present study has been limited to these districts Eighteen diseased samples were collected from 17 locations of which 16 were leaves and two were fruit samples Isolation of the pathogen from infected leaf and fruit samples collected from different locations showed the association of a fungus and the pathogen associated with the disease was found to be *Phytophthora* sp based on cultural and morphological characters Mathew and Beena (2012), reported for the first time *Phytophthora* sp as the cause of nutmeg leaf fall disease Thus, the present findings on the etiology of the disease are in conformity with the earlier report

As a preliminary study, the pathogenicity of first isolate of the pathogen (Mambra), was proved by adopting both culture disc and zoospore inoculation methods under lab condition It showed typical symptoms on leaves, petiole, shoot and fruits as observed in natural condition Likewise pathogenicity of 18 *Phytophthora* isolates was proved on nutmeg seedlings under *in vivo* condition Culture disc and zoospore suspension have been successfully used by several workers to estabhsh the pathogenicity of *Phytophthora* spp on their respective hosts (Turner, 1969, Mehrotra, 1972 and Mammooty, 1978) Among the different methods of inoculation, the culture disc on lower (abaxial) leaf surface with injury recorded early infection as compared to upper surface Since stomatal frequency

and moisture retention was more on abaxial leaf surface, pathogen colonization and penetration were also more prevalent on the abaxial leaf surface (Blodgett and Swart, 2002) Martin (1964) opined that changes in amounts of wax on the leaf surfaces, may also affect pathogen behavior and consequently the infection process Schutte and Botha (2010) were also noticed that inoculation with injury favoured quick symptom expression The symptom expression was found to be delayed with zoospore inoculation in the present study This is in line with the findings of Vilasini (1982) who also observed slow lesion development on leaf when inoculated with the zoospore suspension as compared to culture disc inoculation

Symptoms are the observable effects that a pathogen causes on the growth development and metabolism of an infected host plant Symptomatological studies are important in better understanding of the disease In the present investigation, detailed studies were carried out to understand the symptoms produced by the pathogen on leaves, shoots and fruits both under natural and artificial conditions Field symptom observed under natural condition was the extensive defoliation of green leaves of nutmeg during the South West monsoon period The characteristic symptoms on the leaves were the development of dark brown water soaked lesions mainly on the midrib of the leaves which later enlarged and spread along the lateral veins resulting in blighting Petioles of the leaves showed black discoloration and resulted in premature defoliation Black lesions observed on young shoots led rotting and drying up of shoots from the tip to downwards with die- back symptom The symptoms on fruits appeared as water-soaked lesion on fruit surface led to rotting of the rind and later spread to pericarp, mace and kernel with fluffy mycelial growth on outside and inside the fruits Affected fruits splitted and dropped off prematurely Same type of symptoms on leaves, shoots and fruits of nutmeg were described by Mathew and Beena (2012)

Symptoms observed on artificial inoculation were almost similar to those produced under natural condition Eighteen isolates of the pathogen did not show much variation in symptom expression However, an yellow halo was noticed around the water soaked lesion under artificial condition and the intensity of yellow

halo varied among isolates Initial symptom on leaves was observed within 24 48 h of inoculation and showed premature defoliation of green leaves in 3-5 DAI Initial symptom on shoot initiated within 4 5 DAI and drying up noted after 14-16 days of inoculation Days for initial infection and drying up of shoots slightly varied among the isolates On inoculation on fruits also, variation was observed for initial infection and time required for rotting The earliest infection was produced by PMoF-17, an isolate from fruit collected from Parakkadavu and also showed full rotting in 10 DAI, whereas, other isolates had taken 12 15 days Attempt to study the symptoms on nutmeg seedlings by root inoculation showed drying up of leaves and shoots, without defoliation and the infected plants completely dried up in 30 DAI Death of plants due to root infection of *Phytophthora* sp has been reported by several workers, like drying up of black pepper by *P capsici* (Sarma and Nambiar, 1982), *Citrus* trees by *P citrophthora* (Graham, 1992) and death of oak trees due to *P ramorum* (David and Rizzo, 1999)

The next point of consideration was to study the virulence of the 18 isolates As per the grouping, PPaL-1 and PPaF-17, the isolates (leaf and fruit samples) from Parakkadavu, Thrissur (District) and PSrL-10 from Sreemoolanagaram, Ernakulam (District) were highly virulent ones as they showed the maximum lesion area, per cent leaf fall and early leaf fall which are considered as parameters for high virulence in the present study The isolate PKoL-2 (Kodissery) and PKtL-6 (Koottala), were the least virulent ones and rest 13 isolates were grouped as moderately virulent Thus, this study revealed that, there is variation in virulence among the isolates collected from different locations which may be due to climatic conditions Similarly, Werres *et al* (2001) also observed variability among the different isolates of *P ramorum* collected from different locations on Germany and Netherland

Cultural and morphological characters of the pathogen especially of fungal origin are the important criteria for the exact identification of the pathogen Hence, a detailed study on the cultural and morphological characters of various isolates of the pathogen was carried out on five different media *viz* carrot dextrose

agar, potato dextrose agar, oat meal agar, coconut water agar and V8 juice agar which showed variations

Variation among the isolates in cultural characters was observed only in carrot agar medium Six types of colony characters *viz* white/dull white, sparse/fluffy mycelium with distinct/ weak rosette or lobbed or powdery growth pattern were observed among the 18 isolates Dense cottony white mycelium with pronounced concentric rings growth pattern was observed for all the isolates in PDA Dull white mycelium with intermittent lobbed and white sparse aerial mycelium with stellate growth patterns were observed in oat meal agar and coconut water agar media respectively While in V8 juice agar, all isolates showed appressed white mycelium with weak rosette pattern Colony characters of the different *Phytophthora* sp were studied by several workers Brasier and Griffin (1979) observed sparse aerial mycelium with stellate and striated pattern for *P palmivora* on carrot agar medium Werres *et al* (2001) noticed sparse aerial mycelium with weak rosette pattern in carrot agar, oatmeal agar and V8 juice agar Pronounced concentric rings were formed on V8 and weak ones on carrot agar in case of *P ramorum* Bhai and Sarma (2005) observed lobbed pattern for *P meadii* It also showed rosaceous on carrot agar and stellate on V8 and Rye A agar (Widmer, 2010) Shekhar *et al* (2011) noticed four different growth patterns like cottony, petaloid, rosaceous and stellate for *P capsici* grown on PDA *P citrophthora* showed dense cottony like or rosette or stellate growth patterns in V8 juice agar medium (*Phytophthora* data base) Likewise, *P colocasiae* showed variable characteristics from creeping whitish mycelia with slight zone of striations in V8 agar (Tsopmbeng, 2012)

From this study, it is observed that, *Phytophthora* isolates of nutmeg showed similarity to *P meadii* with respect to rosette, lobbed and stellate colony patterns and to *P ramorum* with regard to weak rosette and concentric ring like cultural characters Eventhough there is consistency in these characters, it was difficult to correlate colony characteristics with other morphological characters of the genus so as to differentiate them into different groups

The growth rate of the different isolates of pathogen studied in the five media did not show much variation among the isolates, but variation was observed among the different media Of the different media tested, oat meal agar was found to be the best one in promoting the growth of the pathogen followed by carrot agar and V8 juice agar was the least effective one

The morphology of an individual is the ultimate expression of its growth processes, final display of all its complex relationships with its normal habitat Identification of a species has been based more on morphology than on any other criterion Therefore the morphological characters such as type of mycelium, type of sporangiophore, sporangial shape, size, L/B ratio and pedicel length and chlamydospores of 18 isolates of the pathogen in different media were studied

The mycelium was branched, coenocytic and hyaline All the isolates produced abundant sporangial characters in all the five media tested No variation was noticed among isolates in sporangial characters on different media The sporangia were borne either terminally or laterally on the sporangiophore in a sympodial fashion, caducous and the diameter of the papilla fall in the range of 4 2 – 8 0 µm that, they were considered as semipapillate (Alizadeh and Tsao, 1985) Sporangia were mostly ovoid or elongated ovoid or ellipsoid, with round base

Caducous nature of sporangia was reported in several *Phytophthora* spp *viz P palmivora* (Brasier and Griffin, 1979), *P meadii* (Stamps, 1985), *P capsici* (Mammootty *et al*, 1991), *P ramorum* (Werres *et al*, 2001) and *P colocasiae* (Tsopmbeng, 2012) However, Mchau and Coffey (1994) reported the non caducous sporangia in *P citrophthora* Most of the *Phytophthora* spp showed variation in sporangial shape According to Waterhouse (1974) and Stamps (1985) *P meadii* produced papillate, ellipsoid or elongated, obpyriform, occasionally spherical shaped caducous sporangia Mchau and Coffey (1994) described the sporangial characteristics of *P citrophthora* as ellipsoid, broadly ovoid, globose, limoniform, or extremely distorted with prominent papillae and semi papillate in some isolates Werres *et al* (2001) also reported semi-papillate mostly 5 8 µm in

diameter, ellipsoid, spindle-shaped or elongated to ovoid and caducous sporangia for *P ramorum* Similar semi papillate sporangia with ellipsoid or ovoid sporangial shape were also observed in *P colocasiae* (Tsopmbeng, 2012)

Considerable variation in sporangial production, size and L/B ratio were observed with 18 isolates on different media All the isolates produced abundant sporangia in all the five media Early sporangial production (24 – 48 h) was observed in carrot agar and potato dextrose agar media and sporangial count was high in oat meal agar and carrot agar media It is also noted that, among the isolates, PPaL-1 (Parakkadavu) showed maximum sporangial production in carrot agar and oat meal agar, PMaL-4 on PDA and PPoL-3 and PDeL-13 in coconut water agar and V8 juice agar media respectively The sporangial count was less with PMoF-18 (Mookkanoor- isolate from fruit)

Hence, considering the overall performance of different media on the cultural and morphological characters, oat meal agar supported faster growth followed by carrot agar With respect to sporangial characters, early sporangial production was observed in carrot agar whereas, maximum sporangial count was noticed in oat meal agar, indicating both oat meal agar and carrot agar media were generally good Thus the present study confirmed the findings of Turner (1969), Waterhouse (1974), Prem (1995) and Veena (1996) who also reported oatmeal agar and carrot agar as the best media for the good growth and sporangial production of *Phytophthora* spp

Size and length to breadth ratio of sporangia were frequently considered as important characteristics in identifying *Phytophthora* species (Ho, 1981, Waterhouse *et al* 1983 and Stamps *et al* 1990) Much variation was observed in sporangial length compared to breadth and L/B ratio among the isolates Among different isolates, PMaL 4 showed maximum sporangial length in oat meal agar and carrot agar whereas, PVeL-5, PKuL-16 and PPaF-17 showed maximum length in PDA, coconut water agar and V8 juice agar media respectively Among different media, maximum sporangial length was observed in oat meal agar, varied from

35 55 to 71 90 µm followed by 32 4 to 49 0 µm in carrot agar Most of the isolates recorded sporangial breadth of 20 36 µm in all the media Maximum L/B ratio of 2 2 was recorded for PMtL 8 in oat meal agar whereas the lowest (1 1) was for PKkL-14 in V8 juice agar The results of the study revealed that, maximum sporangial length and L/B ratio were observed in oat meal agar followed by carrot agar whereas maximum breadth was recorded in coconut water agar followed by oat meal agar and the minimum sporangial length, breadth and L/B ratio of the pathogen were recorded in potato dextrose agar medium

The L/B ratio of isolates of pathogen showed marked variability in different media Hendrix (1967) had also reported that the L/B ratio of the sporangium of *P palmivora* and *P capsici* vary considerably depending upon the substrate used In the present study, maximum L/B ratio was noticed when the isolates were grown in oat meal agar with a range of 1 5 - 2 2 while, minimum in potato dextrose agar ranged from 1 2 - 1 5

Sporangial dimensions of different *Phytophthora* spp were studied by several workers Mammootty *et al* (1991) conducted detailed studies on the morphological characters of six black pepper isolates of *Phytophthora* on carrot agar medium According to them, the length of sporangia varied from 20 3 to 92 3 µm and breadth from 19 6 to 52 2 µm with the L/B ratio of 1 1 to 2 9 Mchau and Coffey (1994) studied the sporangial dimensions of *P citrophthora* and reported that, sporangial length and breadth varied from 23 to 90 µm long x 18 to 60 µm wide and with L/B ratio ranged from 1 3 to 1 81 On carrot agar medium, *P ramorum* produced sporangia of 25 -97 µm length, 14 - 34 µm width and average size of 45 – 65 to 21- 28 µm with L/B ratio ranged from 1 7 to 2 0 (Werres *et al* 2001) whereas sporangia of *P colocasie* showed 40 to 70 µm long x 17 to 28 µm wide with an L/B ratio of 1 92 to 2 50 (Tsopmbeng, 2012) The sporangial dimensions of *Phytophthora* isolates of nutmeg showed much similarity with the characters of *P meadii* (Waterhouse, 1974, Oudemans and Coffey, 1991) having sporangial length from 20 to 44 µm and breadth from 16 to 29 µm and L/B ratio of 1 3 to 2 0

According to Al Hedaithy and Tasao (1979), length of sporangial pedicel appears to be fixed for a species under normal conditions, and is of high diagnostic value in identification of *Phytophthora* isolates In carrot agar media, the pedicel length of 18 isolates of *Phytophthora* isolates of nutmeg varied from 10 21 to 20 24 µm with an average of 15 3 µm Waterhouse (1974) and Oudemans and Coffey (1991) reported similar range (10 20 µm) in *P meadii*

The presence/absence and size of chlamydospores is one of the important criteria for the identification and separation of fungal species According to Waterhouse *et al* (1983), the significance of chlamydospores for classification in genes as a whole is restricted since in some species chlamydospores are formed only by certain isolates In the present study, all the 18 isolates produced numerous chlamydospores on all the five media tested The presence of abundant chlamydospores were reported in several *Phytophthora* spp *viz P palmivora* (Alizadeh and Tsao, 1985), *P capsici* (Tsao, 1991), *P ramorum* (Werres *et al* 2001) and *P colocasiae* (Misra, 2011) However chlamydospores were rare in *P* meadii (Peries and Fernando, 1966) and *P citrophthora* (Mchau and Coffey, 1994)

The globose, mostly thin walled chamydospores were formed intercalary and terminally in all the media Among different media used, maximum chlamydospores count and diameter were recorded in carrot agar medium with maximum chlamydospores production in PVaL 15 and diameter in PMaL 4 The earlier workers extensively studied the chlamydospore characters of different *Phytophthora* spp Holliday (1980) observed that, most isolates of *P palmivora* produced globose to subglobose chlamydospore with a diameter of 32 42 µm (Holliday, 1980) Tsao (1991) reported globose to subglobose chlamydospore with a diameter of 28 29 µm for *P capsici* Werres *et al* (2001) described the chlamydospore characters of *P ramorum* as globose mostly thin-walled which were formed intercalarlly and terminally, occasionally laterally with a size of 46-60 µm Misra (2011) observed single, globose terminal or intercalary chlamydospores for *P colocasia* with size of 17 38 µm These reports showed that,

chlamydospore characters of *Phytophthora* isolates of nutmeg are more similar with *P colocasiae*

Sexuality is one of the complex area of *Phytophthora* biology The most of the *Phytophthora* species are heterothallic, and produce gametangia only in response to chemical stimulation from an isolate of the opposite mating type (Ko 1978, Brasier 1992) In the present study sexual organ oogonium of the pathogen was observed on carrot agar media, indicating that, these isolates may be heterothalhc

Identification of some *Phytophthora* sp can be difficult due to lack of distinct morphological characters (Leonian, 1934, Brasier, 1971) In addition, morphological traits may overlap between species and such characters can be highly variable and dependent on growing conditions Hence molecular approach has been found to be useful for detailed analysis of genetic variability within and between species Molecular characterisation using Internal Transcribed Spacer regions of rDNA gene repeates was widely used by several workers for the identification of different *Phytophthora* spp (Chowdappa *et al* 2003 Werres *et al* , 2001, Sanker *et* al , 2013) Chowdappa (2011) studied molecular taxonomy of *Phytophthora* sp by ITS RFLP and AFLP analysis and reported ITS analysis as taxonomic marker for the identification *Phytophthora* associated with plantation crops Hence, after the cultural and morphological study of 18 isolates, these were further identified by ribosomal internal transcribed spacer (ITS) sequence analysis

Genomic DNA was extracted from the 18 *Phytophthora* isolates using the GenElute Plant Genomic DNA Miniprep Kit (SIGMA) PCR amplifications of the ITS-1 region were carried out with the primers ITS1 and ITS4 (Chowdappa *et al*, 2003) and yielded a PCR product of nearly 900 bp ITS analysis revealed that, out of the 18 isolates, fifteen isolates showed very close similarity to *P colocasiae* and three isolates *viz* PPoL 3, PMaL-4 and PVaL-15 with *P meadii* However, isolates PPaL-1, PKoL-2, PVeL 5, PKtL-6, PKaL-7, PMtL-8, PThL 9, PTuL 11, PKnL 12, PPaF-17 and PMoF-18 also showed homology with *P citrophthora* In

addition to *P meadii* PPoL 3 and PVaL 15 also showed homology with *P colocasiae* PDeL 13, PKkL-14 and PKuL-16 showed maximum homology with *P colocasiae* followed by *P meadii* While, PMaL-4 also showed homology with *P botryosa* and *P colocasiae* Likewise, PSrL 10 showed homology with *P meadii* and *P botryosa* gene sequences in NCBI databank

The dendrogram consisted of three major clusters of which, the first major cluster was divided into two sub clusters and isolate PPaL-1 formed a single cluster within the sub cluster The other sub cluster again divided to two, of which one cluster include PPaF-17 and second cluster consisted of PKoL-2, PPoL-3, PMaL 4, PKtL 6, PKaL 7, PThL-9, PSrL-10, PKnL 12, PDeL-13, PKtL 14, PVaL-15, PKuL-16 and PMoF 18 In the second major cluster, two sub clusters were observed consisted of PVeL-5 and PMtL-8 The third major cluster consisted of only one isolate PTuL-11 The attempt to find out the relationship between 18 isolates and *P ramorum* using ClustalW tool showed that, isolates PPaL 1, PPaF-17 and PMoF-18 formed a single sub cluster along with *P ramorum* showing very close relationship with each other and all isolates showed 89% similarity with *P ramorum*

The findings of the molecular investigation showed the present investigation showed the possibility of existence of different *Phytophthora* spp *viz P colocasiae P meadii P citrophthora* and *P botryosa* as causal agent of leaf fall disease of nutmeg One possibility is to explain this intra isolate variation is cross breeding which might introduce intra isolate or intra species variation on the rDNA ITS region (Jamal, 2007) Das (2011) reported the variation on the rDNA ITS region among 14 isolates of *Phytophthora* sp collected from the citrus orchards of Maharashtra and data of RFLP analysis suggest 12 isolates as *P nicotianae* and the rest two as *P palmivora* Moreover, Michael (2011) opined that, ITS sequences of several group of genetically closely related species do not allow separation and such species clusters include *P infestans* and sympatric species such as *P mirabilis* and *P phaseoli* and cluster represented by *P meadii*, *P botryosa*, *P colocasiae* and *P citrophthora* Hence it can be a reason for showing homology with different

species of *Phytophthora* Combined application of ITS-1 and ITS 2 analysis may reveal the identity at species level which was beyond the scope of present study

Recalling back the observations on cultural and morphological characters of *Phytophthora* isolates of nutmeg, which were found to be different from *P colocasiae* except in chlamydospore characters and disagreeing with the molecular result in which most of the isolates showed maximum homology with *P colocasiae* Other *Phytophthora* spp which showed similarity in molecular identification were *P meadii*, *P citrophthora* and *P botryosa* in which, *P meadii* showed some phenotypic similarity with *Phytophthora* isolates of nutmeg in growth pattern, sporangial dimensions (length, breadth and L/B ratio) and pedicel length but showed dissimilarity with respect to sporangial shape, papillae, chlamydospore characters However, *P citrophthora* and *P botryosa* did not show any similarity with the phenotypic characters of *Phytophthora* of nutmeg Eventhough *Phytophthora* isolates of nutmeg showed only 89% identity with *P palmivorum*, sum of the phenotypic characters *viz* colony growth pattern, type of papillae, sporangial shape and shape and production of chlamydospores were found to be similar Since, *Phytophthora* isolates obtained from nutmeg plants could not be completely fitted into the cultural, morphological and molecular descriptions of any of these *Phytophthora* spp , a final conclusion could not be derived on the identity of the pathogen

An important aspect in the continuity of disease is the host range of the pathogen Therefore, different hosts including four plantation crops and spices, two ornamental plants and one each of medicinal plant, tuber and fruit crops which are reported to the host of various *Phytophthora* spp were selected in the present study The study revealed that, of the 13 hosts screened, six *viz* rubber, vanilla, rose, *Coleopsis*, *Eucalyptus* and *Citrus* were found to be susceptible to the pathogen and showed the characteristic symptom, as observed on nutmeg leaves It is also observed that, the infection of *Phytophthora* of nutmeg on rubber and vanilla showed characteristic symptoms as that of abnormal leaf fall and Phytophthora rot caused by *P meadii* respectively Plants such as cocoa, black pepper and *Colocasia*

developed necrotic hypersensitive reaction and the pathogen failed to cause infection in arecanut, coconut, cardamom and camboge The findings in the present revealed that, the *Phytophthora* isolates of nutmeg were found to have host range, including rubber, vanilla, rose, *Coreopsis*, *Eucalyptus* and *Citrus* and these host can also serve as a collateral hosts, for the perpetuation of the pathogen

Phytophthora is a destructive pathogen known to have very wide host range and it varies from species to species Bobr-Tylingo (1954) listed the susceptible plants and stated that *P palmivora* principally attacks cocoa, rubber, palms and various citrus fruits in addition to 56 species which are less severely affected Chee (1969) reported *Colocasia*, rubber, cocoa as hosts of *P botryosa* Das (1982) found that, *Phytophthora* isolates of black pepper can cause infection on arecanut, coconut, rubber, cocoa and cardamom Prem (1995) described coconut, rubber, black pepper and *Colocasia* as the hosts of *P palmivora* of cocoa *P capsici* have broad host range including members of very diverse and phylogenetically distinct plant families such as solanaceous, cucurbit and leguminous crops (Erwin and Ribeiro, 1996) Wide host range of *P citrophthora* with 83 genera in 51 families was reported by several workers (Gerlach, 1976, Orlikowski *et al* , 2001, Jamal, 2007, Salamone, 2011) which included *Citrus* spp , strawberry, peach, *Rhododendron*, rubber etc Jain and Sharma (2003) studied the host range of *P meadii* and reported rubber, cocoa, arecanut, cardamom and vanilla as the hosts of the pathogen

The wide host range pathogen, *P ramorum*, was first identified in California from tanoak *(Lithocarpus densiflorus*) and coast live oak (*Quercus agrifolia*) (David and Rizzo, 1999) *P ramorum* attacks plants in 12 families including *Rhododendron* and *Viburnum* (Werres *et al* 2001), different cultivar of *Rosa* spp (Moralejo & Hernandez, 2002), *Eucalyptus* (Brasier *et al*, 2005*)*, *Quercus* spp (Denman *et al*, 2005) and *Citrus limon* and *Citrus deliciosa* (Moralejo *et al*, 2006) Singh (2012) identified *Colocasia*, rubber, and black pepper as host of *P colocasiae* A study conducted by Bindu (2011) with different

Phytophthora isolates of rubber collected from Kerala and Karnataka revealed the association of *P citrophthora* and *P colocasiae* in addition to *P meadii*

Another important aspect of investigation was to find out the cross infectivity of commonly occuring *Phytophthora* spp in Kerala such as, *P palmivora* of coconut and cocoa, *P meadii* of arecanut rubber, cardamom and vanilla, *P capsici* of black pepper, *P colocasiae* of *Colocasia* and *P citrophthora* of *Citrus* on nutmeg On cross inoculation, it is observed that, all *Phytophthora* spp except *P meadii* of rubber caused infection on nutmeg leaves However, the development of characteristic symptom and reisolation was successfull with *P meadii* of vanilla and *P citrophthora* of *Citrus* and hypersensitive reaction was noticed with *P palmivora* of coconut and cocoa, *P meadii* of arecanut and cardamom, *P capsici* of black pepper and *P colocasiae* of *Colocasia* Thus the study, indicated that, nutmeg is a collateral host of *P meadii* of vanilla and *P citrophthora* of *Citrus* and non host of *P palmivora*, *P capsici*, *P meadii* of arecanut, rubber and cardamom and *P colocasiae*

It is interesting to mention that, different isolates of *P meadii* viz rubber, arecanut , cardamom and vanilla and *P citrophthora* of *Citrus* showed infection and characteristic symptoms on rose, indicating rose as a collateral host of these pathogen A search of literature did not give relevant information about the infection of *P meadii* on rose In addition, *Eucalyptus* and *Coreopsis* were also found sensitive to *P citrophthora* but not to *P meadii* of vanilla

Mammootty *et al* (1988) conducted cross inoculation studies with six isolates of *Phytophthora* from six different hosts like cocoa, black pepper, arecanut, coconut, rubber and cardamom and observed positive reaction on all host plants tested and the symptom produced on the respective hosts by different isolates was more or less identical and which was contradictory to the reports of Tucker (1927), Holliday and Mowat (1963) and Chandramohan *et al* (1979) Prem (1995) observed infection of *P palmivora* of cocoa on coconut and rubber and not in arecanut In this context, it is worthwhile to mention the statement of Boccas (1980)

that, "from the more practical point of view, it is of particular interest to note that the crossing of two different species, if occurring in nature, may produce a wide range of progeny, greatly variable in morphology, physiology and pathogenic aggressiveness This might well contribute to the evolution of new *Phytophthora* population and their pathogenic adaptation"

Reading back the results obtained in cultural, morphological and molecular characterisation, host range and cross infectivity studies, it is evident that, eventhough most of the *Phytophthora* isolates of nutmeg showed maximum similarity with *P colocasiae* in molecular study, they varied in cultural and morphological characters Moreover, the host range and cross infectivity studies also showed negative results Hence the possibility of *P colocasiae* being the causal organism of leaf fall of nutmeg has been ruled out Eventhough, *Phytophthora* of nutmeg showed similarity with *P meadii* in cultural, morphological (sporangial dimensions and pedicel length) and molecular characters, this pathogen could develop symptoms only on rubber and vanilla and failed to cause infection in cardamom and arecanut, the other known hosts of *P meadii* Likewise, among different isolates of *P meadii*, only vanilla isolate showed positive reaction on nutmeg Similarly, *Phytophthora* isolates of nutmeg also showed similarity with *P citrophthora* in molecular characters, but varied in both cultural and morphological characters, whereas it showed positive results in host range and cross infectivity studies Eventhough, *Phytophthora* isolates of nutmeg showed only 89 per cent similarity with *P ramorum* in molecular characteristics, it showed similarity in cultural and many of the morphological characters of *P ramorum* Moreover, *Phytophthora* of nutmeg caused infection on rose, *Eucalyptus* and *Citrus*, which are already be the reported hosts of *P ramorum* Extending host range study with *Quercus*, *Viburnum* and *Rhododendron* which are the other hosts of *P ramorum* may also provide a conclusive results in this regards It is also worthwhile to mention that, among these three, only rose was found to be a host of *P meadii* Hence, from the finding discussed so far, the exact identity of

Phytophthora isolates of nutmeg could not be made out as the distinguishing features overlapped among the various *Phytophthora* species

Three *Trichoderma* isolates obtained from nutmeg rhizosphere soil were identified to species level from National Centre for Fungal Taxonomy (NCFT), New Delhi Accordingly, the three isolates were *T viride* (TN-1 and TN-3) and *T harzianum* (TN 2)

Plant disease control aims at prevention or reduction in the incidence or severity of the disease Among various methods, use of chemicals offers comparatively more effectiveness and quick action in prevention or reduction of disease As leaf fall of nutmeg is found severe during rainy season, use of chemicals offer better control of the disease However, the constant use of fungitoxic chemicals may lead to the occurrence of resistant races of pathogen, phytotoxicity and environmental pollution Studies conducted on the use of antagonists have opened a new avenue for the control of plant diseases Besides being safe and non phytotoxic, antagonists are known to be effective against various plant pathogen and enhance plant growth In the present investigation, an attempt was made to find out effect of certain selected fungicides and antagomsts against nutmeg leaf fall pathogen under *in vitro* and *in vivo* conditions

In vitro evaluation of all chemicals tested under the study were found effective against the pathogen However the efficiency varied with the chemicals Complete inhibition of the pathogen was observed with 1% Bordeaux mixture, potassium phosphonate (3ml/l) and the plant activator (3ml/l), the combination fungicide iprovalicarb + propineb at concentrations of 1 5 and 2 0g/l and higher concentrations of copper hydroxide (2g/l), copper oxychloride (2 5g/l) and cymoxaml + mancozeb (2g/l) Copper hydroxide and copper oxychloride at lower concentrations of 1 5 and 2g/l also showed 90 per cent inhibition Similar findings have been recorded by Prem (1995) who observed complete inhibition of *P palmivora* with 1% Bordeaux mixture, 0 2% copper oxychloride and 0 3% potassium phosphonate and also m agreement with the earlier reports of Platt

(1998), Fernandez-Northcote *et al* (2000) and Kirk *et al* (2005) who also noticed the reduction in the growth of *P infestans* with cuprous oxide, copper sulphate, copper hydroxide and copper carbonate under *in vitro* condition and the effectiveness of iprovalicarb fungicide against *Phytophthora* sp was reported by Thind (2011)

Leaf fall caused by *Colletotrichum gloeosporioides* is another serious problem in nutmeg especially during the flushing time As carbendazim was reported to be effective against *Colletotrichum* and mancozeb against the two pathogens, an attempt was made to study the effect of combination fungicide of carbendazim + mancozeb against the both pathogen which recorded only 70 per cent inhibition of *Phytophthora* but showed complete inhibition of *Colletotrichum* However, Sharadaraj (2014) observed cent per cent inhibition of *P palmivora* with this fungicide at 2 5g/l

Trichoderma viride -1, *T harzianum* and *T viride* -2 the isolates from nutmeg and the reference cultures *viz T viride*, *T harzianum* and *Pseudomonas fluorescens* showed antagonistic activity against *Phytophthora* isolate of nutmeg All *Trichoderma* sp showed cent per cent inhibition of the pathogen by overgrowth mechanism and the bacterial antagonist *P fluorescens* (KAU), showed only 61 11 per cent inhibition Efficacy of *Trichoderma* against *Phytophthora* spp have been reported by many workers Bhai (2000) and Vijayaraghavan (2003) observed overgrowth of *Trichoderma* spp on *Phytophthora* and parasitized the pathogen by hyphal lysis, penetration and coiling, besides the production of volatile compounds The effectiveness of *Trichoderma* sp may be due to its fast growth, competitive saprophytic ability, production of secondary metabolites or antibiotic, direct parasitism and lysis thereby checking the growth of the pathogen (Ding, 2010, Vijayan, 2011 and Ambuse, 2015) Rubio *et al* (2000) and Paul (2006) observed 74 and 72 per cent inhibition of *P infestans* and *P capsici* by *P fluorescens* respectively

It is a well established fact that, many chemicals and antagonists which are promising against the pathogen under *in vitro* condition may not be effective in the hosts Hence, it has become pertinent to evaluate the efficiency of the fungicides and antagonists under *in vivo* condition also More than 88 per cent incidence was noticed in all chemical treatments, as most of the leaves have been infected due to artificial inoculation (Fig 3) Whereas in case of bioagents, in which treatments were given as prophylactic, the incidence was comparitvely less, with 41 3 to 46 7 per cent only

On reviewing the effect of treatments on disease severity, all treatments were found superior to control, recording 79 4 – 66 1 per cent disease reduction over control (Fig 4) Among the different treatments, 1% Bordeaux mixture spray + soil drenching of copper hydroxide (2g/l) and 1% Bordeaux mixture spray +soil application of *T viride* showed maximum reduction of disease Shashidhara (2010) reported the efficacy of Bordeaux mixture spray + soil drenching of copper hydroxide in the management of foot rot of black pepper and thus supported our findings Spraying of 1% Bordeaux mixture and soil application of *T viride* has also been reported for the management of *Phytophthora* diseases of black pepper and cardamom (KAU, 2011) It is worthwhile to mention that, spraying of 1% Bordeaux mixture alone also showed better result A spate of literature suggest the effectiveness of Bordeaux mixture against *Phytophthora* diseases (Chandramohanan, 1983, Veena and Sarma, 2000, Mammootty, 2003, Bhai and Sarma 2005) It is also observed that, soil drenching with fungicides containing systemic chemicals *viz* iprovalicarb +propineb and cymoxanil + mancozeb were superior than contact ones However, spraying and drenching of copper hydroxide or copper oxychloride and spraying of Bordeaux mixture + soil drenching of copper oxychloride provided maximum reduction of *Phytophthora* disease in the present study These are in agreement with the findings of Boughalleb (2006) who also reported effective management of *P cactorum* of apple trees with soil drenchmg of cymoxaml + mancozeb and iprovalicarb +propineb Similarly, Mushrif *et al* (2011) also observed the effectiveness of cymoxanil + mancozeb, in the management of

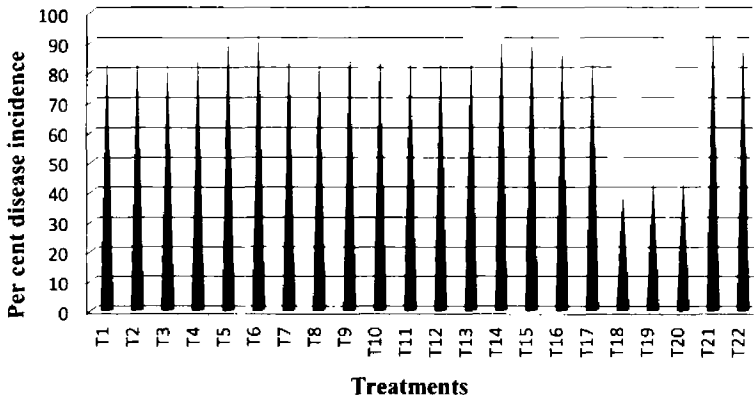


Fig 3 Effect of treatments on per cent disease incidence

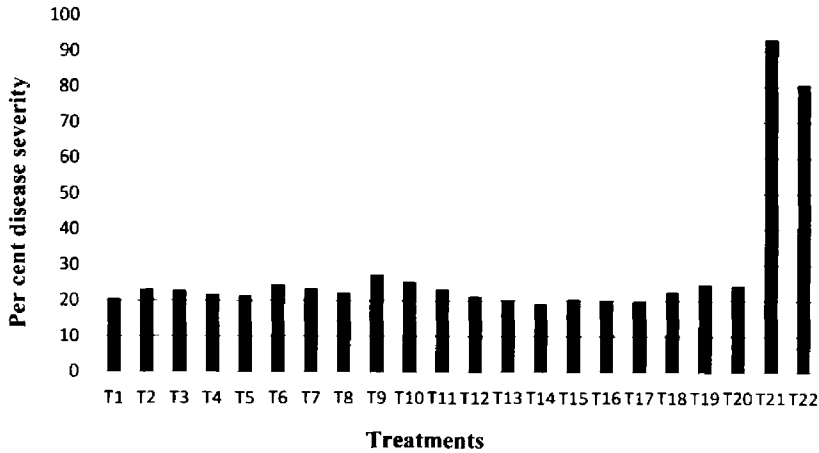


Fig 4 Effect of treatments on per cent disease severity

patch canker of rubber caused by the *Phytophthora* sp under *in vitro* and field conditions A search on literature revealed many reports on the effectiveness of copper fungicides against *Phytophthora* diseases Garbelotto *et al* (2010) also observed copper hydroxide as the most effective fungicide for controlling *P ramorum* and according to Foster and Hausbeck (2010), soil drenching of copper hydroxide (0 2 %) provided better management against *Phytophthora* diseases Similarly, Mammootty, (2011) also suggested management of foot rot of black pepper with 1% Bordeaux mixture as spray and drench and soil drench with copper oxychloride (0 3%)

The combination fungicides carbendazim +mancozeb which was least effective against *Phytophthora* under lab condition also showed better performance in the host system Hence this fungicide can be used if the monsoon extends during August – September months to control both pathogens This is in line with the results of Gupta and Jamal (2010) and Mishra and Pandey (2011) who also reported the effectiveness of carbendazim +mancozeb for the management of *P nicotianae* causing leaf blight and fruit rot of bell pepper and *Colletotrichum capsici* of turmeric respectively Eventhough, soil application of *Trichoderma* alone was found effective, use of both antagonists, as spray of *P fluorescens* and soil application of *Trichoderma* showed better result Similar observation has been recorded by Zegeye (2011) who also noticed spraying of *P fluorescens* along with soil application of *T viride* provide better management of *P infestans* causing late blight of potato Likewise, Ramachandran (2011) also reported the effectiveness of the microbial inoculants like *Trichoderma* and *Psuedomonas* in the reduction of *Phytophthora* infection in many horticultural crops

On reviewing the impact of treatments on disease progression only 0 87 3 33 per cent progress was noticed among the treatments, in which minimum was recorded in 1% Bordeaux mixture spray followed by application of iprovalicarb +propineb Similarly, leaf fall was very less in all treatments and no leaf fall was noticed in the treatments *viz* spraying of cymoxanil + mancozeb / iprovalicarb +propineb , 1% Bordeaux mixture spray + soil drenching of copper hydroxide, spraying

and drenching of copper hydroxide / oxychloride and spraying of copper hydroxide and copper oxychloride

Summing up the findings so far, it is observed that, spraying of 1%Bordeaux mixture + soil drenching of copper hydroxide and spraying of 1%Bordeaux mixture and soil application of *T viride* of KAU showed maximum reduction of leaf fall disease of nutmeg and all copper fungicides especially Bordeaux mixture were found effective against the disease In addition, bioagent, *Trichoderma* also provided effective management of the pathogen Eventhough, spraying and drenching treatments alone were found to be effective against the disease, both spraying + soil application of fungicides/ bioagents showed better result

It is worthwhile to mention that, management of soil borne pathogen is very difficult by using any single control measure Hence, to achieve more effective control with less environmental pollution, it is better to adopt control measures, which combines chemical and biological control strategies in a holistic way rather than using a single component strategy

Summing up the discussion so far, it may be concluded that, the pathogen associated with leaf fall disease of nutmeg is *Phytophthora*, showing similarity to different species of *Phytophthora* in cultural, morphological and molecular characters, with the host range of rubber, vanilla, rose, *Coreopsis*, *Eucalyptus* and *Citrus* and could be managed by chemicals/ bioagents especially copper fungicides and *Trichoderma*

Summary

6. SUMMARY

Nutmeg is unique among tree spices as it is the donor of the two distinct spices, kernel and mace Nutmeg trees are prone to various fungal diseases Recently, a leaf fall disease caused by *Pytophthora* sp has become a serious problem in Kerala during monsoon period The causal organism, *Pytophthora* is a serious pathogen causing diseases in several economically important spices and plantation crops in Kerala *viz* black pepper, cardamom, vanilla, rubber, cocoa, coconut and arecanut leading to heavy yield loss or death of the plants

In view of above facts and considering the importance of the crop and the disease, the present investigation was carried out to study the symptomatology, cultural, morphological and molecular characters of the pathogen, host range and cross infectivity of the pathogen and disease management strategies, which will add to our knowledge about this disease

Isolation of the pathogen from the 18 diseased samples collected from different locations showed the association of a fungus, which was found to be *Phytophthora* sp based on cultural and morphological characters The pathogenicity of these 18 isolates was proved under lab and *in vivo* conditions Inoculation with culture disc on lower leaf surface with injury showed early infection as compared to zoospore suspension

The characteristic symptoms of the disease was the dark brown water soaked lesions on the midrib of the leaves which later spread along the lateral veins to leaf lamina resulted in blighting and premature defoliation Petioles of the infected leaves showed black discoloration Rotting and die back symptoms were observed on young shoots Fruits showed rotting of the pericarp and rind, which later spread to mace and kernel and affected fruits splitted and dropped off prematurely All isolates showed same type of symptoms on various plant parts under both natural and artificial conditions except, the development of yellow halo around the water soaked lesion on artificial inoculation on leaves

Variation in virulence was noticed among the different isolates PPaL-1 and PPaF-17, the isolates (leaf and fruit samples) from Parakkadavu, Thrissur (District) and PSrL 10 from Sreemoolanagaram, Ernakulam (District) were categorised as highly virulent and PKoL 2 (Kodissery) and PKtL 6 (Koottala) as least virulent ones and the other 13 isolates were grouped as moderately virulent

Variation among the isolates in cultural characters was observed only in carrot agar medium which showed six types of colony characters *viz* white/dull white, sparse/fluffy mycelium with distinct/ weak rosette or lobbed or powdery growth pattern Pronounced concentric rings, intermittent lobbed, stellate and weak rosette growth patterns were noticed on PDA, oat meal agar, coconut water agar and V8 juice agar media respectively Variation in the growth rate was noticed only among the different media not with the various isolates

There was no variation among isolates in the sporangial characters on different media The sporangia were borne terminally/laterally on the sporangiophore in a simple sympodial fashion, caducous, semipapillate, mostly ovoid or elongated ovoid or ellipsoid, with round base Variation was noticed among these isolates and media for sporangial production and count Early sporangial production (24 - 48h) was observed in carrot agar and potato dextrose agar media and maximum count was in oat meal agar and carrot agar media Sporangial count was less in PMoF-18 (Mookkanoor isolate from fruit) in all the five media tested

Variation in sporangial breadth and L/B ratio among the isolates were less as compared to sporangial length in different media Among the isolates, PMaL-4 showed maximum sporangial length in oat meal agar and carrot agar whereas PVeL-5, PKuL-16 and PPaF-17 showed maximum length in PDA, coconut water agar and V8 juice agar media respectively Considering the aspects of sporangial dimensions, media and the isolate, the maximum length (71 9 µm), breadth (36 0 µm) and L/B ratio (2 2) were recorded by PMaL-4 in oat meal agar, PKaL-7 in coconut water agar and PMtL 8 in oat meal agar media respectively Maximum sporangial length and L/B ratio were observed in oat meal agar followed by carrot

agar whereas maximum breadth was recorded in coconut water agar followed by oat meal agar and the minimum sporangial length, breadth and L/B ratio were recorded in potato dextrose agar medium Sporangial length varied from 20 2-71 9 µm, breadth 16 4- 36 0 µm with average size of 31 9- 49 5 x 22 3 27 9 µm, L/B ratio 1 4 1 8 in different media The pedicel length among the isolates was 10 2 to 20 2 µm with an average of 15 3 µm

Chlamydospores were globose, thin walled, borne intercalary and terminally In all isolates the maximum count and diameter were observed in carrot agar medium and PVaL-15 isolate showed the maximum production in all the media Oat meal agar and carrot agar were found to be best media for the study of cultural and morphological characters of *Phytophthora*

Comparison of *Phytophthora* isolates of nutmeg with other *Phytophthora* spp such as *P meadii*, *P palmivora*, *P capsici*, *P colocasiae* *P citrophthora* and *P ramorum* showed some similarity to *P meadii*, *P colocasiae* and *P ramorum*

In molecular characterisation of 18 *Phytophthora* isolates, 15 showed maximum homology with *P colocasiae* and three isolates, PPoL-3, PMaL-4 and PVaL 15 recorded maximum homology with *P meadii* Isolates PPaL 1, PKoL 2, PVeL-5, PKtL-6, PKaL-7, PMtL-8, PThL-9, PTuL 11, PKnL-12, PPaF-17 and PMoF 18 also showed homology with *P citrophthora* and PMaL 4 and PSrL 10 also with *P botryosa* The dendrogram of 18 isolates consisted of three major clusters of which, the first major cluster include PPaL-1, PKoL-2, PPoL 3, PMaL 4, PKtL-6, PKaL-7, PThL-9, PSrL-10, PKnL 12, PDeL-13, PKtL-14, PVaL-15,PKuL 16, PPaF 17 and PMoF-18 The second major cluster consisted of PVeL-5 and PMtL 8 and PTuL-11 placed in the third major cluster The dendrogram of *Phytophthora* isolates with ramorum, showed close relationship with PPaL-1, PPaF-17 and PMoF 18

Host range of *Phytophthora* of nutmeg includes rubber, vanilla, rose, *Coreopsis*, *Eucalyptus* and *Citrus* Nutmeg is also a host of *P meadii* of vanilla and *P citrophthora* of *Citrus* and non host of *P palmivora*, *P capsici* *P meadii* of arecanut, rubber and cardamom and *P colocasiae* Rose is found to be a host of *P meadii*

isolates of arecanut rubber cardamom and vanilla and *P citrophthora* of *Citrus* Similarly, *Eucalyptus* and *Coleopsis* are also the hosts of *P citrophthora* of *Citrus* but non hosts of *P meadii* isolates

Considering cultural morphological and molecular characterisation host range and cross infectivity studies the exact identity of *Phytophthora* isolates of nutmeg could not be made out as the distinguishing features overlapped among the various *Phytophthora* species

In *in vitro* screening of chemicals complete inhibition of the pathogen was observed with 1% Bordeaux mixture, potassium phosphonate (3ml/l) and the plant activator (3ml/l) copper hydroxide (2g/l), copper oxychloride (2 5g/l) and the combination fungicides iprovalicarb + propineb (1 5 and 2 0g/l) and cymoxanil ÷ mancozeb (2g/l) *T viride* 1 *T harzianum* and *T viride* 2, the isolates from nutmeg and the reference cultures such as *T viride* (KAU) *T harzianum* (IISR) showed cent per cent and *Pseudomonas fluorescens* (KAU) showed 61 11 per cent inhibition of pathogen

In *in vivo* experiment, all fungicides plant activator and bioagents provided better management of leaf fall disease of nutmeg Among the treatments spraying of 1%Bordeaux mixture + soil drenching of copper hydroxide (2g/l) and spraying of 1% Bordeaux mixture and soil application of *T viride* of KAU were the most effective ones All copper fungicides tested under study were effective against *Phytophthora* of nutmeg of which Bordeaux mixture was the most promising one followed by copper hydroxide and copper oxychloride In addition either spraying or drenching with fungicides containing systemic chemicals *viz* iprovalicarb +propineb and cymoxanil + mancozeb also showed good result Soil application of *Trichoderma* was found to be effective and prophylactic spray of 2% *P fluorescens* and soil application of *T viride* provided better result Eventhough spraying and drenching treatments alone were found to be effective against the disease both spraying + soil application of fungicides/ bioagents showed better result

The findings of the present study turned out to be a boon to the nutmeg growers of the state as it provides better understanding and strategies for effective management of leaf fall disease

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Appendices

APPENDIX –I

COMPOSITION OF MEDIA USED

1. Carrot Agar

Carrot	- 200 g
Agar - Agar	- 20 0 g
Dextrose	- 20 0 g
Distilled water	- 1000 ml

2. Potato Dextrose Agar

Potato	- 200 g
Agar - Agar	20 0 g
Dextrose	20 0 g
Distilled water	- 1000 ml

3. Oat meal Agar

Oats	- 100 g
Agar- Agar	- 15 0 g
Distilled water	- 1000 ml

4. Coconut water agar

Coconut water	- 200 ml
Agar- Agar	- 20 0 g
Dextrose	20 0 g
Distilled water	- 1000 ml

5 V8 Juice Agar

V8 vegetable juice	- 200 ml
Agar Agar	- 20 0 g
Distilled water	- 800 ml

PHENOTYPIC AND MOLECULAR CHARACTERISATION OF *Phytophthora* sp. INCITING LEAF FALL OF NUTMEG

By

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ABSTRACT OF THE THESIS

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ABSTRACT

The study on 'Phenotypic and molecular characterisation of *Phytophthora* sp inciting leaf fall of nutmeg' was conducted in the Department of Plant Pathology, College of Horticulture,Vellanikkara during 2014-2015 The major objectives were to study the cultural, morphological and molecular characters and variability of different isolates of *Phytophthora* sp associated with leaf fall of nutmeg and also to study the host range of the pathogen and to chalkout suitable management strategies

Isolation of the pathogen from 18 samples from different locations revealed the association of the fungus, *Phytophthora* sp and its pathogenicity was established under lab and *in vivo* conditions Inoculation of the pathogen with culture disc on injured lower leaf surface showed early infection than that with zoospore suspension Symptoms observed on leaves, shoot and fruits were almost same under both natural and artificial conditions Variation in virulence was noticed among the isolates collected from different locations The isolates, PPaL-1 and PPaF-17, from Parakkadavu, Thrissur and PSrL 10 from Sreemoolanagaram, Ernakulam were highly virulent PKoL 2, the isolate from Kodissery was less virulent and other 14 were moderately virulent

Cultural and morphological characters of the isolates of pathogen were studied with different media *viz* carrot agar, potato dextrose agar, oat meal agar, coconut water agar and V8 juice agar Variation in cultural characters among the isolates was observed only in carrot agar and the variation in growth rate was noticed among the different media Morphologically, mycelia of *Phytophthora* isolates from nutmeg were branched, coenocytic and hyaline and the sporangia were borne terminally /laterally on the sporangiophore in sympodial fashion, caducous, semi papillate, ovoid/elongated-ovoid/elhpsoid in shape with average size of 31 9 49 5 x 22 3 – 27 9 µm, L/B ratio of 1 4 – 1 8 and pedicel length of 10 21 – 20 24 µm Early sporangial production was noticed in carrot agar and potato

dextrose agar and the maximum count was in oat meal agar and carrot agar Numerous chlamydospores were observed in all media

Comparison on the cultural and morphological characters of *Phytophthora* isolates of nutmeg with other *Phytophthora* spp such as *P meadii*, *P palmivora*, *P capsici*, *P colocasiae* *P citrophthora* and *P ramorum* revealed that, *Phytophthora* isolates from nutmeg could not be completely fitted into the phenotypic description of any of these known *Phytophthora* species However, they showed some similarity to *P meadii*, *P colocasiae* and *P ramorum*

In molecular characterisation, out of 18 isolates of nutmeg *Phytophthora*, 15 showed maximum homology with *P colocasiae* and three *viz* PPoL-3, PMaL-4 and PVaL-15 with *P meadii* Isolates PPaL-1, PKoL-2, PVeL-5, PKtL-6, PKaL-7, PMtL-8, PThL 9, PTuL-11, PKnL -12, PPaF-17 and PMoF-18 also showed homology with *P citrophthora* and PMaL-4 and PSrL 10 with *P botryosa*

Host range of *Phytophthora* isolate of nutmeg includes, rubber, vanilla, rose, *Coreopsis*, *Eucalyptus* and *Citrus* Nutmeg is also a host of *P meadii* of vanilla and *P citrophthora* of *Citrus* and non host of *P palmivora*, *P capsici*, *P colocasiae*, *P meadii* of arecanut, rubber and cardamom Rose is also found to be a host of *P meadii* isolates of arecanut, rubber, cardamom, vanilla and *P citrophthora* of *Citrus*

The cultural, morphological and molecular characters, host range and cross infectivity studies of various *Phytophthora* isolates could not revealed the exact identity of these isolates, as the distinguishing features overlapped among the various *Phytophthora* species

In vitro evaluation of chemicals / bioagents showed complete inhibition of the pathogen with 1% Bordeaux mixture, copper hydroxide (2g/l), copper oxychloride (2 5g/l), potassium phosphonate (3ml/l), combination fungicides, iprovalicarb + propineb (1 5 and 2 0g/l), cymoxanil + mancozeb (2g/l) and

Trichoderma viride -1, *T harzianum* and *T viride* 2, the isolates from nutmeg and the reference cultures *viz T viride* (KAU) and *T harzianum* (IISR)

In *in vivo* experiment, all treatments were superior to control of which, spraying of 1% Bordeaux mixture + soil drenching of copper hydroxide (2g/l) and spraying of 1% Bordeaux mixture and soil application of *T viride* showed maximum reduction of the disease In addition, spraying and drenching of copper hydroxide and copper oxychloride were also found equally effective