CHARACTERIZATION AND DOCUMENTATION OF DISEASES OF PASSION FRUIT

(*Passiflora edulis* Sims.)

By

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(2018-11-016)

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KERALA, INDIA
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THESIS

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COLLEGE OF HORTICULTURE

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KERALA, INDIA

2020

DECLARATION

I, Karthika Mohan (2018-11-016) hereby declare that this thesis entitled **"Characterization and documentation of diseases of passion fruit (*Passiflora edulis* Sims.)"** is a bonafide record of research work done by me during the course of research and that the thesis has not been previously formed for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Certified that the thesis entitled **"Characterization and documentation of diseases of passion fruit (*Passiflora edulis* Sims.)"** is a record of research work done independently by **Ms. Karthika Mohan** (2018-11-016) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, fellowship or associateship to her.

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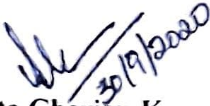

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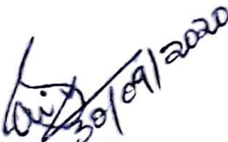

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Introduction

1. INTRODUCTION

Passion fruit (*Passiflora edulis* Sims.), a native of Brazil is an emerging high value crop bearing fruits of great export potential. It is a perennial, vigorous, climbing, woody vine which is valued for its fruits with unique flavor, aroma as well as for its nutritional and medicinal properties. The fruits are rich in vitamin A, vitamin C, potassium, fibre and other nutrients. The tender leaves of the plants are used as leafy vegetable and as a substitute for tea. The leaves and roots possess medicinal values too. The crop is gaining wide popularity among the health conscious new generation because of its antioxidant richness, high dietary fibre content and anti-inflammatory properties.

The crop belongs to the family *Passifloraceae* which includes about 550 species under 12 genera. Among various genera, *Passiflora* is one of the most important genus constituting more than 400 species. *Passiflora edulis f. edulis* (purple passion fruit), *P. edulis f. flavicarpa* (yellow passion fruit), *P. ligularis* (urucu passion fruit), *P. mollisima* (curuba passion fruit), *P. quadrangularis* (melon passion fruit), *P. alata* (sweet passion fruit), *P. cerulae* (blue flower passion fruit) and *P. laurifolia* (lemon passion fruit) are the most important ones among the 60 passion fruit species which bear edible fruits (Manicom *et al*., 2003). Among these, *P. edulis* is the predominant species and its center of origin is South Brazil. The most popular and commercially cultivated varieties of passion fruit include purple passion fruit (*P. edulis f. edulis* Sims.) and yellow passion fruit (*P. edulis f. flavicarpa* Deg.).

Passion fruit is grown mostly in tropical and subtropical regions where the temperature is hot and humid and up to 2100 m altitude. The optimum temperature required for optimum yield ranges from 20 to 30°C. Global production is estimated to be about 8.52 lakh tons and the major producing countries include Brazil, Mexico, Ecuador, Australia, Zimbabwe, Kenya and Columbia (ITI Tropicals, 2007). Currently, South America is the largest producer of passion fruit in the world. The other important passion fruit producers are Peru, New Zealand, South Africa and Hawaii (Fischer and Rezende, 2008). About 95 per cent of passion fruit cultivation in the world comprises

of yellow passion fruit variety and this is mainly used for juice extraction while purple variety is preferred in the fresh market.

In India, the cultivation is confined to the regions of Kerala, Nilgiri hills and Kodai Kanal of Tamil Nadu and North Eastern states like Mizoram, Nagaland, Manipur and Sikkim with an area of 9.11 thousand ha and production of 45.82 thousand tons (Tripathi, 2011). Currently, the crop is being cultivated commercially in the state of Himachal Pradesh also. Manipur has highest production of passion fruit in India followed by Nagaland (Thokchom and Mandal, 2017). The average productivity in India is 5.02 tons/ha which is too low compared to the average productivity of 30-35 tons/ha in other countries like Brazil (Joy, 2010).

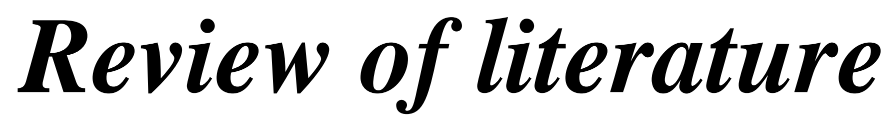
As mentioned earlier, passion fruit is a perennial, herbaceous and woody plant with a vine like growth habit. Biotic problems like diseases can seriously reduce the productivity of the crop leading to the failure of commercial cultivation. Diseases of bacterial, fungal and viral etiologies are reported in passion fruit. Among the diseases, passion fruit woodiness, bacterial spot, root and collar rot, fusarium wilt, anthracnose and scab are the most important ones. Woodiness, bacterial spot, anthracnose and scab affect the quality of fruit and cause severe loss under favourable environmental conditions when proper management practices are not opted. Wilt, root and collar rot can wipe out the entire crop resulting in the ultimate failure of this perennial crop due to negligence in taking suitable control measures.

During the recent past, there is tremendous increase in the cultivation of passion fruit, particularly in the state of Kerala. This is because of its importance as an assured source of income for small and marginal farmers of the state. Due to its peculiar aroma and flavouring properties, fruits are used for the large scale production of nutritious juice. Since the vine is of perennial nature, once the crop is established, the farmer gets an assured income for about six to seven years. There exists a great potential for boosting the production of passion fruit in Kerala. The warm humid climate prevailing in Kerala with its high rainfall is well suited for the cultivation of this crop in the state. But, the longevity and productivity of passion fruits are often challenged by many biotic factors especially diseases (Fischer and Rezende, 2008). Even though

many diseases are reported internationally, no systematic documentation has been conducted in the country particularly in Kerala due to the recent introduction of this crop in the agricultural scenario of the state.

Hence, the present study was undertaken to identify the diseases affecting passion fruit cultivation in Kerala, documentation of the same and characterization of associated pathogens. The study was focused on the following objectives.

- Survey on the occurrence of diseases of passion fruit
- Isolation of pathogens and pathogenicity studies
- Characterization and identification of pathogens
- Symptomatology studies under natural and artificial conditions
- *In vitro* evaluation of fungicides and biocontrol agents against major fungal path



Review of literature

2. REVIEW OF LITERATURE

Passion fruit *(Passiflora edulis)* is a vine species cultivated commercially across the globe for its sweet and juicy fruit. It is an attractive, nutritious fruit crop highly appreciated for fresh consumption and industrial purposes because of its diverse uses for juice, jelly and ice cream products. The crop is a native from Southern Brazil through Paraguay and Northern Argentina (Morton, 1987). In sixteenth century, Spanish Christian Missionaries illustrated the crucifixion of Christ with the help of this flower due to its unique morphological peculiarities and depicted that corona refers to the crown of thorns, three stigmas to the nails at the cross and so on. Hence, the English prefix "Passion" is derived from the passion for Christ (Davidson, 2014). Commercially important varieties of passion fruit are purple passion fruit (*P. edulis f. edulis* Sims.) and standard yellow passion fruit (*P. edulis f. flavicarpa* Deg.).

The peel and juice of yellow varieties of passion fruit contain prunasin and other cyanogenic glycosides (Chassagne *et al*., 1996) and all the varieties of passion fruit are rich in polyphenol content (Ramaiya *et al*., 2013). According to USDA nutrient data (2013), raw passion fruit has water (73%), carbohydrates (22%), protein (2%) and fat (0.7%) and 100 gram amount of fresh passion fruit contains Daily Value (DV) of vitamin C (36 %), dietary fibre (42%), B vitamins; riboflavin (11%) and niacin (10%), iron (12%) and phosphorus (10 %). The crop is being cultivated widely in tropical and subtropical countries of the world. But the establishment and expansion of the crop has been hindered by various biotic and abiotic stresses, the major one being the incidence of disease. The literature pertaining to the diseases affecting passion fruit is reviewed in this chapter.

2.1 OCCURRENCE OF DISEASES

Reports on occurrence of diseases in passion fruit dates back to 1940s. Pathogens belongs to all groups like fungi, bacteria, viruses and phytoplasma were reported to infect the crop. When not properly managed, such diseases lead to economic loss to the farmers by reducing the yield and economic life span of the crop.

2.1.1 Fungal diseases

Pathogenic fungi are responsible for about 30 per cent of all crop diseases (Jain *et al*., 2019). Passion fruit hosts many fungal pathogens which provoke severe losses of agricultural and horticultural crops every year. A perusal of the literature revealed that several diseases with fungal etiology were reported in passion fruit which include diseases affecting above ground plant parts, root and collar region. The diseases caused by fungi reported so far in passion fruit, their occurrence and year of report are summarised in Table 2.1

Table. 2.1 Occurrence of different fungal diseases in passion fruit

Sl. No.	Disease	Year of First Report	Country/Region	Reference
1.	Brown spot	1939	Australia	Smith, 1939
2.	*Septoria* blotch	1939	Peru	Sydow, 1939
3.	*Sclerotinia* rot	1944	Australia	Blackford, 1944
4.	*Fusarium* wilt	1951	Austarlia	McKnight, 1951
5.	Root rot	1970	New Zealand	Young, 1970
6.	Rust	1971	Brazil	Albuquerque, 1971
7.	Collar rot	1976	Uganda	Emchebe and Mukiibi, 1976
8.	Damping off	1978	Australia	Inch, 1978
9.	*Lasiodiplodia* rot	1989	United States	Farr *et al*., 1989
10.	Anthracnose	1991	Brazil	Yamashiro, 1991
11.	*Phomopsis* rot	1992	Mauritius	Lutchmeah, 1992
12.	Scab	1995	Venezuela	Rondon *et al*., 1995
13.	Flower rot	1998	Brazil	Goes, 1998

2.1.1.1 Diseases affecting above ground parts

Fungal diseases were found to infect the crop from the seedling stage till maturity and several authors have reported the occurrence of various fungal pathogens affecting the above ground parts like leaves, stem, fruits, flowers *etc*. Under favourable conditions *viz.* hot and rainy weather, anthracnose causes up to 80 per cent yield or crop loss (Torres, 1983). Anthracnose is reported to occur in all crop growing areas and is considered as the most important disease in passion fruit (Yamashiro, 1991; Cedeno *et al*., 1993; Lutchmeah, 1993; Wolcan and Larran, 2000). The pathogen associated was found to harm *Passiflora edulis*, *P. edulis f. flavicarpa*, *P. alata*, *P. laurifolia*, *P. mollisssima*, *P. quadrangularis and P. ligularis* (Manicom *et al*., 2003).

Scab also known as *Cladosporium* rot was reported in Australia (Simmonds, 1932) and Brazil (Bitancourt, 1935). *Cladosporium oxysporum* was reported to be the causal agent of this disease in Zimbabwe (Bates, 1954) and Australia (Persley, 1993). Later, it was reported in Venezuela by Rondon *et al*. (1995). *C. cladosporoides* and *C. herbarum* were the two pathogens associated with scab in Brazil (Barreto *et al*., 1996). The pathogen caused the death of seedlings in nurseries as well as in the field caused drying of twigs and also affected the commercial quality of fruits. Goes (1998) reported that this disease was observed in all Brazilian passion fruit producing areas causing significant damages when not properly managed.

Septoria blotch was another foliar disease which was firstly reported in Peru and the causal agent is *Septoria passiflorae* (Sydow, 1939). In the same year *Septoria fructigena* was also found as a causal agent for the disease from passion fruits in Kenya (Nattrass, 1939). Later the disease was reported from New Zealand (Dingley, 1959) and Australia (Inch, 1978). *Septoria passifloricola* was a third species of *Septoria* reported from Venezuela, South Africa by Punithalingam (1980). The disease was also reported from other parts of globe like Brazil (Yamashiro, 1991), Mauritius (Lutchmeah, 1993), USA (Alfieri *et al*., 1994) and Colombia (Trujillo *et al*., 1994). Three species of *Septoria* were reported to cause disease in *Passsiflora* species.

Brown spot, another fungal disease found in passion fruit was first reported in Australia by Smith (1939). The incidence of this disease reached 100 per cent in

Venezuela and Hawaii (Aragaki *et al.*, 1969). Passion fruit juice production was reduced by 70 per cent in Kenya between 1966 and 1967 due to this disease (Ondieki, 1975). He revealed that, the most important diseases of passion fruit in Kenya were brown spot caused by the fungus *Alternaria passiflorae* Simmonds and the woodiness virus. The occurrence of the disease was reported from India in 1977 by Ram *et al.*

Puccinia scleriae, the causal agent of rust produced yellow pustules on leaves and twigs which later become brown was firstly reported in Brazil (Albuquerque, 1971) and later in Panama (Esquivel and Labrador, 1977). The disease leads to heavy defoliation followed by death of twigs. Apart from *P. edulis*, the disease was also reported in *P. glandulosa*, *P. cyanea*, *P. rubra*, *P. serrato-digitata*, *P. suberosa*, *P. tricuspis* and *P.tuberosa* (Liberato, 2002).

Gerlach (1980) and Poltronieri *et al.* (1999) reported that *Rhizoctonia solani* (*Thanetophorus cucumeris*) as the causal agent of leaf spots with light green colour having yellow halos. They also reported that the fungal mycelium and microsclerotium were visible on the lesions.

Lasiodiplodia theobromae was found to infect the twigs and caused darkening of phloem and bark tissues which leads to the wilt and drying of plants (Farr *et al.*, 1989). It was reported in passion fruit species such as *P. edulis f. flavicarpa* and *P. quadrangularis* (Farr *et al.*, 1989; Fischer *et al.*, 2007).

Complete plant death and twig wilt was caused by *Sclerotinia sclerotiorum* due to rotting of collar region and the affected twig resulted in considerable damages during colder months in Austarlia (Blackford, 1944). White cottony mycelium and dark sclerotium were developed over the lesions under high humidity conditions (Kagiwata, 1990).

In *P. edulis* and *P. edulis* f. *flavicarpa*, *Phomopsis* rot caused by *Phomopsis tersa* was reported to affect leaves, twigs, especially fruits and resulted in 40 per cent damage to the crop production (Sutton, 1980). The pathogen was responsible for postharvest losses where the pathogen enters the fruit mainly through the cut in the stalk or through skin injuries (Lutchmeah, 1992). *Rhizopus stolonifer,* the rot pathogen

infected the flower bud of passion fruit and this resulted in the putrid nature of flowers and abscission (Manicom *et al*., 2003).

2.1.1.2 Collar and Root Diseases

Diseases affecting root and collar region of the crop are fatal since these can lead to ultimate loss of the crop since the entire plant is affected due to infection. McKnight (1951) reported wilt disease caused by *Fusarium* for the first time in Austarlia which affected the purple passion fruit commercial orchards. The occurrence of the disease was also reported from Brazil (Carvalho and Carvalho, 1968), Panama (Esqivel and Labrador, 1977) and South Africa (Grech and Rijkenberg, 1991) and the disease affected *P. edulis*, *P.foetida*, *P. mollissima* and *P. ligularis* (Gardner, 1989).

Fusarium solani was identified as a serious pathogen causing collar rot disease of *Passiflora edulis* f. *flavicarpa* which results in reduced productivity and crop migration. It was reported to occur in various countries like Uganda (Emchebe and Mukiibi, 1976), Taiwan (Lin and Chang, 1985), USA (Ploetz, 1991), Zimbabwe (Cole *et al*., 1992) and China (Li *et al*., 1993). The disease has been reported in *P. edulis*, *P. edulis f. sp. flavicarpa*, *P. alata*, *P. ligularis*, *P. maliformis* and *P. quadrangularis* (Ssekyewa *et al*., 1999; Junqueira *et al*. 2005).

Root rot caused by *Phytophtora cinnamomi* in New Zealand (Young, 1970) and *Phytophthora nicotianae* in India (Ullasa and Sohi, 1975) is an important disease of passion fruit. *Rhizoctonia solani* (*Thanetophorus cucumeris*) caused damping off diseases in nursery seedlings, root and collar rot in adult plants (Inch, 1978; Bezzera and Oliveira, 1984; Farr *et al*., 1989).

2.1.2 Bacterial diseases

Bacteria belonging to different groups were reported as pathogenic to passion fruit in different countries across the globe. Bradbury (1986) revealed that *Xanthomonas axonopodis* pv. *passiflorae* was responsible for leaf lesions and death of plants, *Pseudomonas syringae* pv. *syringae*, *P. syringae* pv. *passifllorae* and *P. viridiflava* caused leaf spots, *Agrobacterium tumefaciens* caused tumors, *Erwinia carotovora* ssp. *carotovora* caused soft rot and *Ralstonia solanacearum* was responsible for vascular wilt.

Bacterial leaf spot caused by *Xanthomonas axonopodis* pv. *passiflorae* was observed as a serious issue in all passion fruit producing areas in Brazil (Pereira, 1969). It is the most important bacterial disease of passion fruit due to high susceptibility of economically important cultivars and was also observed in Australia (Bradbury, 1986) and Colombia (Castilho and Granada, 1995).

Bacterial grease spot by *Pseudomonas syringae* has been reported in South Africa (Baigent and Starr, 1963), New Zealand (Doepel, 1965) and Australia (Bradbury, 1986).

Table 2.2 Occurrence of bacterial diseases in passion fruit

Sl. No.	Disease	Pathogen	Region/ Country	Year	Reference
1.	Leaf spot	*Xanthomonas axonopodis* pv. *passiflorae*	Brazil	1969	Pereira, 1969
			Australia	1986	Bradbury, 1986
			Colombia	1995	Castilho and Granada, 1995
2.	Grease spot	*Pseudomonas syringae*	South Africa	1963	Baigent and Starr, 1963
			New Zealand	1965	Doepel, 1965
			Australia	1986	Bradbury, 1986

2.1.3 Viral diseases

Several viruses associated with passion fruit were reported by various researchers. Virus belong to the genus *Potyvirus* had been reported to cause disease in passion fruit by various researchers from different parts of the world. Passion fruit woodiness virus (PWV) was the first *Potyvirus* reported to infect passion fruit in Australia (McKnight, 1953). The virus was later reported from Nigeria (Martini, 1962), Taiwan (Chang, 1992) and Japan (Iwai *et al.*,1996). Based on serological and biological properties, PWV was confirmed as the causal agent of woodiness disease from different states of Brazil. But, recent molecular studies of several Brazilian isolates of this

Potyvirus revealed that this is a strain of cowpea aphid borne mosaic virus (CABMV) (Barros *et al.,* 2007).

Cucumber mosaic virus was first confirmed in passion fruit by Teakle *et al.* (1963) from California with electron microscopy and observed CMV virions in *P. caerulea* and *P. alata-cerulae*. The first report of association of CMV with passion fruit woodiness in Australia was given by Taylor and Kimble (1964) while dealing with mixed infections. Brand and Wechmar (1993) reported the association of CMV with a *Potyvirus* (a strain of CABMV) in woodiness affected plants from South Africa.

Passiflora infection with *Passiflora* latent virus was firstly described in 1960s by Brandes and Wetter (1963) in *P. caerulea* and *P. suberosa* in Germany. Similar type of infection was found in sterile hybrid of *P. incarnata* x *P. cincinnata* in Florida, USA by St. Hill *et al*. (1992) and from germplasm collections in UK and Netherlands (Hicks *et al*., 1996). The complete nucleotide sequence of PLV was published later and suggested that it belongs to the genus *Carlavirus* (Spiegel *et al*., 2007).

Passion fruit yellow mosaic virus was another virus reported from Brazil belongs to the genus *Tymovirus* which exhibited characteristic bright yellow mosaic, yellow net and leaf crinkle (Crestani *et al*., 1986). This virus was later reported from Colombia by Morales *et al*. (2002).

Infection of *Passsiflora* spp. with different isolates of *Tobamovirus* was reported from India (Mali and Vyanjane, 1980). Purple granadilla mosaic virus (PGMV) producing mild or line pattern like mosaic was found to infect *P. edulis* in Brazil (Chagas *et al*., 1984). Kitajima *et al*. (1986) reported Passion fruit vein clearing *virus* in orchards of Brazilian growing areas with symptoms like vein clearing, reduced leaf size and fruit size that leads in severe reduction of yield. Brown *et al*. (1993) described a *Geminivirus* designated as *Passiflora* leaf mottle virus resulted in severe curling, distortion and mottling of leaves and fruits with reduced yield and fruit quality. Passion fruit green spot virus belonging to the family *Rhabdoviridae* caused severe damage to passion fruit production in the state of Sao polo and Brazil (Kitajima *et al.*, 1997).

Table 2.3: Occurrence and distribution of viral diseases of passion fruit

Sl. No.	Virus	Year of Report	Place/Region	Reference
1.	Passion fruit woodiness virus	1953	Australia	McKnight, 1953
		1962	Nigeria	Martini, 1962
		1992	Taiwan	Chang, 1992
		1996	Japan	Iwai *et al*., 1996
2.	Cucumber mosaic virus	1963	California	Teakle *et al*., 1963
		1964	Australia	Taylor and Kimble, 1964
		1993	South Africa	Brand and Wechmar, 1993
3.	Passiflora latent virus	1963	Germany	Brandes and Wetter, 1963
		1992	Florida, USA	St. Hill *et al*. 1992
		1996	UK	Hicks *et al*., 1996
		1996	Netherlands	Hicks *et al*., 1996
4.	Passion fruit yellow mosaic virus	1986	Brazil	Crestani *et al*., 1986
		2002	Colombia	Morales *et al*., 2002
5.	Tobamovirus	1980	India	Mali and Vyanjane, 1980
6.	Purple granadilla mosaic virus	1984	Brazil	Chagas *et al*., 1984
7.	Passion fruit vein clearing virus	1986	Brazil	Kitajima *et al*., 1986
8.	Passiflora leaf mottle virus	1993	Puerto Rico	Brown *et al*., 1993
		2003	Brazil	Novaes *et al*., 2003
9.	Passion fruit green spot virus	1997	Brazil	Kitajima *et al*., 1997

2.2 SYMPTOMATOLOGY

The review of studies on the symptomatology of various diseases of passion fruit caused by fungi, bacteria and virus are given below.

2.2.1 Leaf Spots

A glance through the literatures revealed that *Cladosporium, Alternaria, Septoria, Colletotrichum, Rhizoctonia* were the major fungal genera causing leaf spot disease in

passion fruit and the bacterium mainly responsible for leaf spot is *Xanthomonas axonopodis* pv. *passiflorae*

Cladosporium sp. mainly affected young tissues of leaves and produced small round spots of 3 – 6 mm diameter which on the initial stages appeared as translucent and later became necrotic. These lesions caused perforations on the leaves and deformation when infected on veins (Bitancourt, 1935).

Alternaria passiflorae produced reddish brown spots on leaves of 5 mm diameter which grow larger reaching 2 cm size with zonations under high humidity conditions (Brien, 1940). Thin mass of black spores covering the middle of lesions were seen predominantly on the abaxial surface of leaves (Holliday, 1980).

In the case of *Septoria* blotch, leaves were the most affected parts showing light brown slightly round necrotic spots of around 4 to 10 mm in diameter normally encircled by a chlorotic halo (Louw, 1941). Abscission occurs even due to a single lesion on the leaf and when the disease reaches 15 – 20 per cent of the leaves in the same plant, complete leaf abscission occurred (Yamashiro, 1991; Piza Jr. (1994).

Peirera (1969) described that bacterial leaf spot caused by *Xanthomonas axonopodis* pv. *passiflorae* was characterized by defined angular leaf spots which were translucent, dark green and anasarcous encircled by a clear chlorotic yellow halo and during favourable conditions the lesions become bigger and coalesces leading the leaves to wilt and fall.

Esquivel and Labrador (1977) observed that *Puccinia scleriae* caused yellow pustules on leaves and twigs which later become brown and high rust severity caused intense defoliation.

Colletotrichum gloeosporoides, the anthracnose pathogen produced small round light spots that later turned to brown spots with 1 cm diameter and these spots coalesced to form large dried areas and the center of spots became brittle and fall apart (Yamashiro, 1991). Goes (1998) reported that all aerial organs of the passion fruit were infected by this pathogen.

Poltronieri *et al.* (1999) reported that passion fruit leaves infected with *Rhizoctonia solani* produced rounded small light green round waterish spots with yellow halo, which increased in size and coalesced forming irregular necrotic and the fungus hypha grew on the lesions and extended very quickly to healthy tissue, as leaves were held together by a mycelial thread, on which micro sclerodia was formed.

According to Baigent and Starr (1963), *Pseudomonas syringae,* the causal agent of grease spot produced numerous angular spots of necrotic tissues with chlorotic halos around the spots and also form light-brown, slightly-sunken cankers which resulted in girdling and killing of terminal and lateral shoots. The pathogen also produced typical symptoms of small water soaked areas of dark-green colour on the immature fruit which later develop to circular or irregular, tan to dark-brown spots of greasy texture, essentially with superficial necrotic lesions having sharply-defined margins and subsequently a hardened crust or shield was formed and this crust was inhabited by a number of different microbes.

2.2.2 Fruit rots

According to Inch (1978), the infection of *Septoria* occurred at any stages of development of fruits and produced circular lesions with well-defined borders with black punctuations at the center corresponding pycnidia of pathogen that may reach great extents on fruits affecting maturation and development, however the damages were only concerned with the fruit skin.

Brown spot caused by *A. passiflorae* produced circular spots on mature fruits when they were half way through the growth process which were sunken reddish brown of about 1 to 3 cm diameter affecting the pulp and damaging the commercial value of the fruit (Fullerton, 1982) whereas *Alternaria alternata* produces spots on fruits with dark green-greasy margins (Manicom *et al*., 2003).

According to Farr *et al*. (1989), *Lasiodiplodia theobromae* produces light brown spots which later became darkened and covered with fungus and pycnidium prior to the soft rot of fruits.

Phomopsis rot was one of the postharvest fruit rot of passion fruit and the causal agent was reported as *Phomopsis tersa.* The infection initiates after two to three days

of harvest showing suppression and collapse of tissue around the stalk turning to brown. Later white mycelial growth become visible over the fruits and by the tenth day of infection the whole fruit become affected damaging the endocarp and seeds (Lutchmeah, 1992).

Fischer and Rezende (2008) revealed that *C. gloeosporoides*, the anthracnose pathogen initiates the symptoms on fruits as cork like layer on the surface of fruit with a sunken appearance and as the fruits matures, round dark spots of 1 cm were formed often with acervuli at the center. Later these spots extend largely over the fruit affecting the quality of fruit and result in premature fruit drop. Light brown lesions were initially produced superficially and then become sunken and greyish brown which on progression enlarges to about one cm diameter and the fruit skin becomes papery and acervuli were formed at the centre of spots (Joy and Sherin, 2012)

2.2.3 Floral diseases

Infection of *S. passiflorae* initiates on the calyx region of flowers and reaches the peduncle resulting in early flower drop (Louw, 1941). Manicom *et al.* (2003) reported that *Cladosporium* sp., the scab pathogen when infected on flowers produced small round spots on bud sepals and peduncles which appeared translucent initially, later became necrotic and caused perforations. He also stated that *Rhizopus stolonifer,* the rot pathogen produces dark spots that are water soaked on the interior of the flower bud especially on the sepals and petals which spread to entire flower bud with the production of dark grey mycelia bearing sporangia. Kwon *et al.* (2016) reported that the symptoms of grey mold in passion fruit caused by *Botrytis* species were appeared on flowers, which turned brown and then died, with masses of grey or brownish spores produced on infected tissues.

2.2.4 Root and collar diseases

According to Manica *et al.* (1981), *Fusarium* wilt of passion fruit typically affects the xylem vascular system, leading to the impermeability of vascular walls and preventing the translocation of water to other plant parts and under high relative humidity conditions lesions and fissures could be found in the plant collar and stems which may be confused with the root collar symptoms.

S. scleortiorum affected the collar region and twigs of passion fruit causing the plant death and wilting of affected twig and under high humidity conditions white cottony mycelium and darkened sclerotium were developed over the lesions (Kagiwata, 1990).

The symptoms of root and crown rot caused by *Phytophthora* sp. were observed as mild chlorosis followed by plant wilt, defoliation and death due to the rotting of root and collar region which exposed the plant cortical tissue (Cole *et al.*, 1992)

In the case of collar rot caused by *Fusarium* sp., the first aboveground symptom appeared as mild dieback followed by changing of leaf colour to pale green, leaf wilt, defoliation and finally plant death resulting from the necrotic girdling of the plant collar (Cole *et al.*, 1992).

Arogundade *et al*. (2018) reported that passion fruit plants with passion fruit woodiness virus infection showed symptoms like symptoms such as systemic mosaic, mottling leaf distortion, upward curling and puckering of lamina were observed in three farms in Oyo state.

2.3 INFLUENCE OF WEATHER PARAMETERS ON DISEASE

Weather parameters like rainfall, relative humidity, temperature *etc.*.. have an influence on the development and spread of disease. Congenial environmental conditions favours the infection on susceptible host by the virulent pathogen (Disease Triangle). Blackford (1944) revealed that the rot pathogen, *S. sclerotiorum* caused significant damages during colder months in Australia. The per cent disease intensity of wilt disease caused by *F. oxysporum* f sp. *passiflorae* was favored by high temperature and relative humidity and the crop grown in sandy soil was more prone to the disease (Kiely and Cox, 1961). The per cent disease severity of passion fruit scab disease was higher during spring time when the temperature was mild and the optimum temperature for the causal agents varied from 19.5 - 24°C for *Cladosporium oxysorum*, 20 - 28°C for *C. cladosporoides* and 28 - 30°C for *C. herbarum* (Domsch *et al.*, 1980). Average temperature of 27°C and long raining periods were ideal condition for the development of anthracnose caused by *C. gloeosporoides* and the incidence of disease was low during winter season (Piza Jr, 1994). The germination of maximum number of

conidia of *C. gloeosporoides* occurred between 30 and 33°C in dark and was accelerated between 22 and 25°C in the presence of light (Neto *et al*., 1994). Piccinin *et al*. (1995) reported that the bacterial spot disease of passion fruit caused by *X. axonopodis* pv. *passiflorae* was more severe under high temperature and relative humidity during which the incubation period was shorter generally lasting 5 to 10 days. According to Goes (1998), flower rot occurs mainly in summer during the periods with prolonged rain and loses upto 63 per cent was noted in *P. alata* from Brazil. *Phytophthora* root and crown rot was frequently observed in clay and acid soils during the rain periods when the temperatures vary between 26° and 30°C (Ploetz *et al.*, 2003). The per cent incidence of brown spot caused by *Alternaria sp.* on yellow passion fruit in high rainfall areas could be as high as 98 % and the disease occurs on fruits during the rainy season whereas disappeared during the dry season (Junquiera *et al.*, 2005).

2.4 ISOLATION AND PATHOGENICITY STUDIES

Isolation of pathogen, the first step which is essential for establishing the pathogenicity and for characterization of the associated pathogen of any disease. Carrot disc method (Moller and DeVay, 1968) was followed for the isolation of *Ceratocystis fimbriata* associated with fruit rot symptoms of passion fruit in the region of Tanhaçu, Brazil from naturally infected branches were made by Firmino *et al*. (2013). The discs of carrots were taped together and incubated in a moist chamber at 25 ± 2°C for one week. After sporulation of the fungus on the carrot tissue, the ascospore masses formed at the tip of the perithecia were transferred to malt extract agar medium (MEA). The fungal pathogens associated with grey mold disease of passion fruit was isolated by excising diseased flower tissues from the margins of lesions followed by surface sterilization in 1% NaOCl for 10 s and rinsing three times with sterile distilled water. The bits were then placed on water agar and cultured at 25°C for 2 days and the mycelial tips of developing fungal cultures were then transferred to potato dextrose agar (PDA) for identification (Kwon *et al*., 2016).

The preliminary confirmation method for plant pathogens was given by Robert Koch, Friedrich and Loeffler. It is the technique used to prove whether a particular pathogen is responsible for disease development and is collectively called as Koch's postulates.

Artificial inoculation studies revealed that wounding has profound effect on collar rot development and Fischer *et al.* (2005) reported that the symptoms were reproduced only when the roots or collar of plants were injured before inoculation.

Kwon *et al*. (2009) prepared the inoculum of *S. rolfsii* from mycelial mats grown for seven days on PDA and the fruits were inoculated with 100 g of the inoculum source near the basal end of fruit in order to prove the pathogenicity and the growth of mycelium appeared 4 days after inoculation which later developed to severe fruit rot.

Two inoculation methods were evaluated for the establishment of pathogenicity of *C. fimbriata* by Firmino *et al*. (2013). In the first method, disc of fungal mycelium (1 cm) from 10-day-old colonies was placed on the wounds of four injured passion fruits and a disc was applied to an injured fruit as a control. For the second method, a needle was used to injure the mesocarp of four fruits and 25 µl drop of inoculum (10^8 cylindrical endoconidia/ml) was applied to the wounds and a drop of water was applied to a wounded fruit as a control. The inoculated fruits were incubated in hermetically closed plastic boxes at 25°C and high humidity (>90%), and subjected to alternating light/dark photoperiods for six days. They reported that the symptoms of rot caused by *C. fimbriata* were observed about six days after inoculation with both methods and no infection was observed in the controls.

For the establishment of pathogenicity of *R. solani*, Rahayu (2014) conducted detached leaves assay and for this healthy soybean trifoliate leaves from five weeks old plants were surface sterilized with five per cent of NaOCl for five seconds. Sterilized leaves were rinse twice using sterile distilled water. Sterile samples were placed on sterile dish contain sterile filter paper, then moistened with sterile distilled water. Each leaflet was artificially inoculated with a five mm diameter mycelia disk from seven days *R. solani* plate culture. Another leaves were kept healthy without inoculated as control in separate dish.

Pathogenicity test of *Botrytis*, the causal agent of grey mold in passion fruit was carried out by Kwon *et al*. (2016) on the flowers of two-year-old passion fruit plants grown in pots. Five flowers from one passion fruit plant were inoculated by spraying a conidial suspension of 3×10^5 conidia/ml until run-off. Five negative control flowers

of another passion fruit plant were treated with sterilized distilled water. The two plants were stored in a moist chamber with more than 90 per cent relative humidity at 25°C, and after two days the plants were placed in a greenhouse. They reported that after seven days after inoculation, gray mold symptoms similar to those observed in the field developed on the inoculated flowers, whereas the control flowers remained asymptomatic.

Gil (2017) prepared the inoculum of *P. nicotianae* var. *parasitica*, *C. gloeosporoides sensu lato* and *P. drechsleri* quantified in a Neubauer chamber, dissolved in an agar-water solution of 2 per cent (w/v) and five drops of 20 µl of this inoculum were placed on healthy fruits and incubated. In case of *Phytophthora* spp., the mycelia grew in PDA media incubated under an incandescent light and a suspension of 3000 sporangia per ml and for *C. gloeosporoides* sensu lato, the myceliagrew in the PDA and a suspension of $1x10^5$ conidia/ml was prepared. Each pathogen was inoculated in five fruits and the results revealed that the symptoms were reproduced and the isoaltes of *P. nicotianae* var. *parasitica and P. drechsleri* were the microorganisms that induced the appearance of symptoms in the shortest average time (12.15 and 17.8 days respectively) and the shortest time for sporulation was observed in the isolate *P. nicotianae* var. *parasitica* (23.1 days).

Vico *et al*. (2017) established the pathogenicity of *Diplodia* sp. in which fruits were surface sanitized and wound inoculated with mycelial plugs (5 mm in diameter) from 10days old cultures grown on PDA. Three control fruit each were inoculated with uncolonized PDA plugs were also kept. After 7 days incubation in plastic containers (RH 90 to 95%) at room temperature (25°C), brown lesions developed on inoculated fruit, while wounded, uninoculated, control fruit remained symptomless.

Aslam *et al*. (2017) conducted pathogenicity assessment of *Ralstonia solancearum* in tomato varieties in order to differentiate resistant cultivars. They followed the protocol of transferring three-week-old seedlings individually to polythene bags and one week after transplantation, the plants of each cultivar were inoculated with 30 ml of bacterial culture containing $1x10^7$ cfu/ml through soil drenching. One by third of root system of each cultivar was slightly injured by inserting a sharp sterilized knife about 2 cm away from the stem prior to drenching to facilitate penetration of the

bacterium. The symptoms appeared 4 days after inoculation. The symptoms were first observed on leaves and then progressed toward other parts of plants resulting in complete wilting in susceptible cultivars within 14 days.

Mycelium plugs of the isolate *Colletotrichum gloeosporoides* (Pafc1) were inoculated on the leaves of yellow passion fruit (*P. edulis* f. *flavicarpa*) and cultivar Tainung No. 1 by Chen and Huang (2018). They revealed that typical anthracnose symptoms appeared, and setose acervuli were observed on leaves of yellow passion fruit at 3 days post inoculation, whereas the acervuli were only produced when the leaves turned yellow and similar results were also obtained by spraying a spore suspension (10^5 spores/ml).

Lima *et al*. (2019) reported that the artificial inoculation with *Fop* (*Fusarium oxysporum* f. sp. *passiflorae*) involved application of a spore suspension of 10^6 conidia mL^{-1} and infestation of the potting media with *Fop* grown in sand and cornmeal substrate. In order to establish the pathogenicity of *Fusarium oxysporum* f. sp. *passiflorae,* conidial spore suspension of $1x10^6$ spores/ml concentration was prepared and inoculated on six months old *P. edulis* seedlings with 12 true leaves and the symptoms were observed within three weeks and included wilt, chlorotic yellow leaves with subsequent defoliation and necrotic roots (Garcia *et al.* , 2019).

Pathogenicity of the isolates of *Alernaria* sp. causing spot was demonstrated by Guevara *et al*. (2019) on inoculating symptomless surface-sterilized cherimoya leaves using agar plugs with mycelium (5 mm^2) from two different isolates obtained from single-spore cultures. Two pieces of mycelium were placed on the surface of each leaf, one on the central vein and other on the leaf edge, using two leaves per isolate and a control. Leaves were incubated in a moist chamber at 25°C. Infected leaves showed black lesions with concentric zonation after one week of incubation.

2.5 CHARACTERISATION OF PATHOGENS

Characterisation of pathogens was done based on cultural, morphological and molecular characterisation.

2.5.1 Fungi

The fungal pathogens were identified up to the genus level based on cultural and morphological characters. Cultural characters include colour, texture, growth rate, pattern of growth, sporulations, pigmentation, colour on the back side of petri plates and presence of fruiting bodies. The various microscopic observations like colour of hyphae, branching pattern, sepatation, presence of conidia, conidial septation, types, shape and size of spores, length by breadth ratio of spores and presence of sexual structures were coming under the morphological characters.

2.5.1.1 Cultural and Morphological Characterisation

Cladospoporium oxysporum produces macronematous, straight or slightly flexuous conidiophores that are nodulose, pale or mid pale brown, smooth and up to 500 µm long and 3-5 µm wide with terminal and intercalary swellings of 6-8 µm diameter (Ellis, 1971). Conidiphores of *C. cladosporoides* and *C. herbanum* were olivaceous brown and bear conidia from the upper to middle portion (Domsch *et al*., 1980).

Fusarium wilt caused by *Fusarium oxysporum* f. sp. *passiflorae* produced micro and macro conidia of dimensions 5-16 x 2.4 – 3.5 µm and 27 – 55 x 3.3- 5.5 µm respectively (Gerlach and Nirenberg, 1982). The colonies were fast growing with sparse to abundant aerial mycelium with white, pink or salmon coloured pigmentation (Nelson *et al*., 1983).

According to Hanlin (1990), *Haematonectria haematococca* (anamorph: *Fusarium solani*), causal agent of collar rot of pathogen produced single or groups of reddish perithecia after two weeks in culture medium of about 200 µm in diameter and unitunicate asci of 80 µm size in which eight bicellular ascospores of 14 µm length.

Phytophthora nicotianae, the causal agent of *Phytophthora* root and crown rot forms non-caducous ellipsoid, ovoid, pyriforme to spherical sporangia with usually a single papillum (Erwin and Ribeiro, 1996).

The causal agent of Anthracnose of passion fruit, *Glomerella cingulata* (anamorph: *Colletotrichum gloeosporoides*) produced whitish to grey coloured

colonies with thick to sparse lawns of aerial mycelium (Jeffries *et al.*, 1990). The conidia were hyaline, one celled 7-20 x2.5-5.0 µm and either cylindrical with obtuse ends or ellipsoidal with a rounded apex and narrow, truncate base and this fungus is heterothallic and its teleomorph can be readily induced in culture medium with the formation of perithecia which are sub spherical, dark brown to black, 90-220 µm in diameter containing hyaline unitunicate asci (Wolcan and Larran, 2000). Similarly, *C. gloeosporoides* isolates from avocado, mango, papaya and passion fruit produced cylindrical conidia with rarely on end pointed and the size ranged from 9.6-20.6 µm x 3.4-8.2 µm (Peres *et al.*, 2002).

Septoria passiflorae and *Septoria passifloricola*, the causal agents of blotch of passion fruit leaves can be easily distinguished by the conidial length *i.e. S. passiflorae* has conidia of 35-52 x 1.5- 2 µm size while *S. passifloricola* has 14-22 x 1.5- (2-2.5) µm size length (Cline, 2007).

A. passiflorae produces solitary conidia on its host and chains of conidia up to five in culture (Ellis, 1971). Nine species of *Alternaria* have been described as pathogens to passion fruit i.e. *A. passiflorae* (Holliday, 1980), *A. alternata* (Goes, 1998) and the less common species were *A. macrospora*, *A.aliena*, *A. aragakii*, *A. hawaiiensis*, *A. tenuissima*, *A. tropica*, *A. guangxiensis*, *A. bannaensis* and *A. tomato*. (Chen and Zhang, 1977; Ram *et al.*, 1977; Simmonds, 1993 and Manicom *et al.*, 2003). The conidia produced were ovoid, obclavte, obpyriform with 18-63 µm x 7-18 µm size often have three to eight transverse septa and one - two longitudinal septa (Ploetz *et al.*, 2003). On PDA *A. alternata* produced creamy to ashy white submerged mycelia with entire margin, circular shape and concentric rings (Nagrale *et al.*, 2013).

S. rolfsii, the rot fungi grew between 10°C and 35°C and optimal temperature for the growth was 30°C on PDA. The white mycelium usually formed many narrow hyphal strands in the aerial mycelium which were 3 to 8 µm in width and after 4 days typical clamp connection structure were formed (Kwon *et al.*, 2009). After 20 days, they examined sclerotium for the characteristics and stated that small, uniformly sized, globoid sclerotia were produced in great numbers. The sclerotia were initially white then turned to dark brown at maturation and the maximum numbers of sclerotia were produced at 25 to 30°C and the size of sclerotia were 1 to 3 mm.

Black, globose perithecia (237.30 x 233.70 µm) with long rostrum (423.30 µm) were observed by Firmino *et al.* (2013) in association with fruit rot disease in Brazil. The ascospores were hat-shaped with dimensions of 3.98 x 5.26 µm and asexual aleurioconidia were brown (12.43 x 9.50 µm) and two types of endoconidia were observed; cylindrical (17.80 x 3.93 µm) and doliform (7.95 x 6.89 µm), in chains. Thus they confirmed that the morphological characteristics indicated that the fungal isolate was of the genus *Ceratocystis.*

Norhayati *et al.* (2016) reported that *L. theobromae*, the black rot pathogen produced vigorously growing mycelium on PDA with aerial mycelia grew uniformly in all directions and fully covered the surface of the media within 3 to 4 days. The colour of the colony changed gradually from light grey after four to seven days of incubation to black after 2 weeks of incubation and the bottom part of the fungus only became darker after 3 weeks of incubation. They also reported that subsequently, the fungus produced stromata and pycnidia and the pycnidia that were produced were initially soft but hardened when the culture matured at 4 weeks and the culture sporulated only after 4 weeks of incubation. The morphological characters described by them includes that the immature conidia were non septate, thick cell-walled, oval in shape and hyaline and mature conidia were observed to be septate, oval-shaped and brown in colour with the presence of irregular longitudinal striations.

Five *Botrytis* isolates were recovered from the grey mold infected plant samples of passion fruit by Kwon *et al.* (2016). They reported that all fungal colonies of isolates were grey brown, produced sclerotia on PDA and the conidia were one-celled, mostly ellipsoid or ovoid, colourless or pale brown, 6 to 19 × 4 to 12 µm (n = 50) in length and conidiophores were 15 to 33 µm long.

2.5.2.2 Molecular Characterisation

Firmino *et al.* (2013) characterised the fungal pathogen associated with Fruit rot symptoms in Brazil and found that The ITS sequence (GenBank Accession No. JX477136) of the nuclear rDNA was 99 per cent identical to that of *C. fimbriata* strain Ayy316 (HQ529711) isolated from pomegranate in Sichuan Province, China (Xu *et al.*, 2011).

To confirm the identity of the fungus associated with Grey mold disease in passion fruit, Kwon *et al.* (2016) amplified the complete internal transcribed spacer (ITS) rDNA region of a representative isolate MHGNU F114 from South Korea using ITS1/ITS4 primers (White *et al.* 1990). The DNA products were cloned into the pGEM-T Easy vector (Promega, Madison, WI) and the resulting plasmid (pOR187) was sequenced using universal primers at Macrogen Services (Daejeon, South Korea). BLASTn search conducted by them for the ITS rDNA sequence of the isolate MHGNU F114 (GenBank Accession No. KU234690) confirmed that the sequence obtained was homologous and shared 99 per cent identity with the *B. cinerea* isolate LGM002 from Brazil and clone GTB from Florida, causing gray mold on pea (KC683713) and German thyme (KT737373), respectively (Dallagnol *et al.* 2014). For further confirmation, two nuclear protein-coding genes were sequenced: heat-shock protein 60 (HSP60) and DNA-dependent RNA polymerase subunit II (RPB2) (Staats *et al.*, 2005). The HSP60 and RPB2 sequences (KU760985 and KU760986) of the isolate were 99 to 100 per cent identical to those of *B. cinerea* strains B05.10 and T4, respectively.

Molecular characterization of the EF-1α region of *Fusarium oxysporum* f sp. *passiflorae* was performed by Garcia *et al.* (2019). The BLAST analysis of the amplicon of isolate MK430070 showed 100 per cent similarity with an isolate of *F. oxysporum* f. sp. *passiflorae* from North America (JF332039) and 99 per cent similarity with an isolate from Australia (KX434919). A portion of the β-tubulin gene was also amplified, sequenced with primers Bt2a and Bt2b (MK430071) and the analysis of the amplicon showed 99 per cent similarity with an isolate from Brazil (AF008540).

2.5.1 Bacteria

Xanthomonas axonopodis pv. *passiflorae,* causal agent of bacterial leaf spot of passion fruit was a rod shaped 0.5 µm x 1.5 µm, gram negative and aerobic bacteria which does not form spores or capsules and has a polar flagellum (Bradbury, 1986). In culture medium, these bacteria produced bright yellow colonies which are bright yellow, mucous, round and convex (Almeida *et al.*, 1994).

According to Baigent and Starr (1963), *Pseudomonas syringae* - causal agent of bacterial grease spot of passion fruit was a gram negative, rod shaped bacteria with an

average size of 1.2-3.2 µm in length and 0.2-0.5 µm in width and were capable of producing capsules but endospore production was absent. These bacteria gave positive reaction for acid production test and all the isolates were capable of producing a typical green-fluorescent, water-soluble pigment.

2.5.3 Virus

Nattrass (1944) described a method for transmitting the virus of the 'woodiness' disease of passion fruit by means of single leaf grafts. The grafts can be made from one species to another in the genera *Passiflora* and *Tacsonia* and from these genera to species of *Adenia* and vice versa.

Electron microscopical analysis conducted by Chiemsombat *et al.* (2014) for the morphological characterisation of Passion Fruit Severe Mosaic Disease in Thailand using leaves with typical symptoms revealed *Potyvirus*-like flexuous rod particles, ca. 750 nm long and pinwheel inclusion bodies in the cytoplasm of infected cells.

Sap transmission of virus associated with mosaic, blistering, curling, and necrosis symptoms of passion fruit was carried out by Yeturu *et al.* (2018). For this ten seedlings of Cucurbita pepo (squash) were inoculated with sap from symptomatic passion fruit leaves using 0.01 M phosphate buffer (pH 7.0). Vein clearing and blistering symptoms were observed 11 days post inoculation.

To confirm infectivity of Passion fruit woodiness virus Arogundade *et al.* (2018) collected leaf samples from symptomatic plants were used as a source for mechanical sap inoculation onto 21-day-old healthy passion fruit seedlings maintained in an insect-proof screen house and observed that newly emerging leaves of inoculated plants developed mild chlorotic mottle symptoms approximately 14 days after inoculation.

2.6 DISEASE MANAGEMENT

Applications of fungicides like benzimidazole, cupric, dithiocarbamate, chlorothalonil and tebuconazole were effective against anthracnose disease of passion fruit (Phelps, 1991). Rocha and Oliviera (1998) revealed that *Trichoderma* spp. can be effectively used to control the postharvest loss by this pathogen. The fungicides

prochloraz and imazalil show the best results for the control of postharvest rots (Benato *et al*., 2002). Fungicides quoted as efficient against the scab disease of passion fruit caused by *Cladosporium* sp. were tebuconazole, strobilurin, copper oxychloide, mancozeb, captan, chlorothalonil + copper oxychloride (Willingham *et al*., 2002). Amata *et al*. (2013) conducted greenhouse trials at the National Agricultural Research Laboratories, Kenya to determine the effectiveness of pruning, coupled with the use of various fungicides including Bavistin (carbendazim), Contaf (hexaconazole), Topsin (thiophanate methyl), Ridomil (metalaxyl) and biocontrol agents (*Trichoderma harzianum* and *T. asperellum*) in controlling dieback disease of passion fruit. Treatments were applied either as sprays or paste on the affected area. All treatments had a significant effect in controlling dieback relative to the control. Carbendazim, hexaconazole and *T. harzianum* were the most effective treatments, irrespective of the application method. *T. harzianum* performed better than *T. asperellum* while thiophanate methyl was better than metalaxyl.

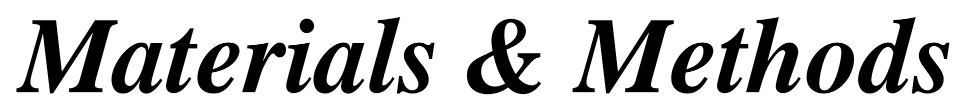
Benzimidazoles are effective against *Septoria blotch* of passion fruit but it was already verified that some of the *Septoria* isolates were resistant to them. Therefore, use these fungicides as a mixture or alternate with other group of fungicides having different mode of action (Peterson, 1977). Thiabendazole or Thiophanate-methyl + chlorothalonil when applied at 15-day intervals showed to be efficient in controlling this disease (Piza Jr., 1994).

A study was initiated by Ssekyewa *et al*. (1999) to develop a sustainable, environment-friendly collar rot control package in passion fruit crop. The potential of chemical control was assessed and it was found to be economical only in the case of copperoxychloride applied as a drench at a rate of 60 g/20 l of water. Other fungicides tested were Rovral 70 WP (iprodione) and Cercobin 50 L (thiophanate methyl). According to Fischer *et al.* (2005), application of fungicides effective against oomycetous organisms directly applied on the plant collar soon after the beginning of the rainy season may control the collar and root rot caused by *Phytophthora nicotianae*.

Studies showed mancozeb + iprodione are effective at controlling the Brown spot disease under high humidity conditions (Menzel *et al*., 1989) According to Willingham *et al*. (2002) recommended that copper compounds, carbamates and

strobilurins applied at 7 to 14 days intervals from the onset of symptoms and at greater intervals when conditions are less favourable controls Brown spot caused by *Alternaria* sp.

Ribeiro *et al*. (2017) studied the effect of pyoverdine, a xiderophore produced by fluorescent *Pseudomonads* on the population of *X. axonopodis* pv. *passiflorae*, the causal agent of bacterial blight of passion fruit. It was found that the filtrate containing pyoverdine of higher concentration ($Abs_{363} = 0.231$) reduced the number of cells of *X. axonopodis* pv. *passiflorae* and significantly reduces the severity of bacterial blight.


Materials & Methods

3. MATERIALS AND METHODS

The project entitled "Characterization and documentation of diseases of passionfruit (*Passiflora edulis* Sims.)" was carried out in the Department of Plant Pathology, College of Horticulture, Kerala Agricultural University, Vellanikkara during 2018-2020. The details of materials used and methodologies followed during the course of study are described in this chapter.

3.1 SURVEY ON THE OCCURRENCE OF DISEASES OF PASSION FRUIT

Purposive sampling surveys were conducted in different passion fruit growing tracts of Kerala to assess and document various diseases affecting the crop cultivated in different districts of the state. Five districts *viz.* Thrissur, Ernakulam, Palakkad, Wayanad and Idukki were selected and details of the locations of survey in each district are given in Table 3.1 and Plate 1. From each field, samples were collected for further studies and symptoms on each plant part was closely examined and documented. Each type of symptom was catalogued by assigning a specific code pertaining to that location.

3.1.1 Cataloguing of diseased samples

The symptomatic plant parts collected from each location were catalogued and abbreviated as leaf spot (LS), leaf blight (LB), wilt (Wt), fruit rot (FR), bud blight (BB) and leaf malformation (LM) which were prefixed with the abbreviations assigned for each location (Table 3.2). Accordingly, the various locations were abbreviated as MNP for Manjapra (Angamaly), VKA for Vellanikkara, MDK for Madakkathara, KAN for Kannara, PAZ for Pazhayannur, KLY for Kolazhy, MVP for Muvattupuzha, NEL for Nelliyampathy, NEN for Nemmara, AMB for Ambalavayal and THN for Thankamani.

3.1.2 Collection of infected samples

During the survey, the infected plants were tagged and each part of such infected plants were closely observed to record the symptoms produced under natural, field conditions. The plant samples showing different types of symptoms were collected separately in polythene sample covers, labelled properly and brought to the laboratory for further investigation.

Table 3.1: Details of locations of purposive sampling survey

Sl. No.	District	Place	Agro Ecological Unit (AEU)	Geographical co-ordinates
1.	Thrissur	Vellanikkara	Northern central laterites (AEU 10)	10.5359/ 76.188
		Madakkathara	Northern central laterites (AEU 10)	10.5612/ 76.2624
		Kannara	Northern central laterites (AEU 10)	10.5357/ 76.3353
		Kolazhy	Northern central laterites (AEU 10)	10.5752/ 76.2179
		Pazhayannur	Palakkad central plains (AEU 22)	10.6832/ 76.4334
2.	Palakkad	Nelliyamapathy	Southern high hills (AEU 14)	10.5348/ 76.694
		Nemmara	Palakkad central plains (AEU 22)	10.6017/ 76.6116
3.	Ernakulam	Muvattupuzha	South central laterites (AEU 9)	9.983/ 76.5798
		Manjapra	South central laterites (AEU 9)	10.2192/ 76.4637
4.	Wayanadu	Ambalavayal	Wayanad central plateau (AEU 20)	11.619/ 76.21
5.	Idukki	Thankamani	Southern high hills (AEU 14)	9.8398/ 77.0371

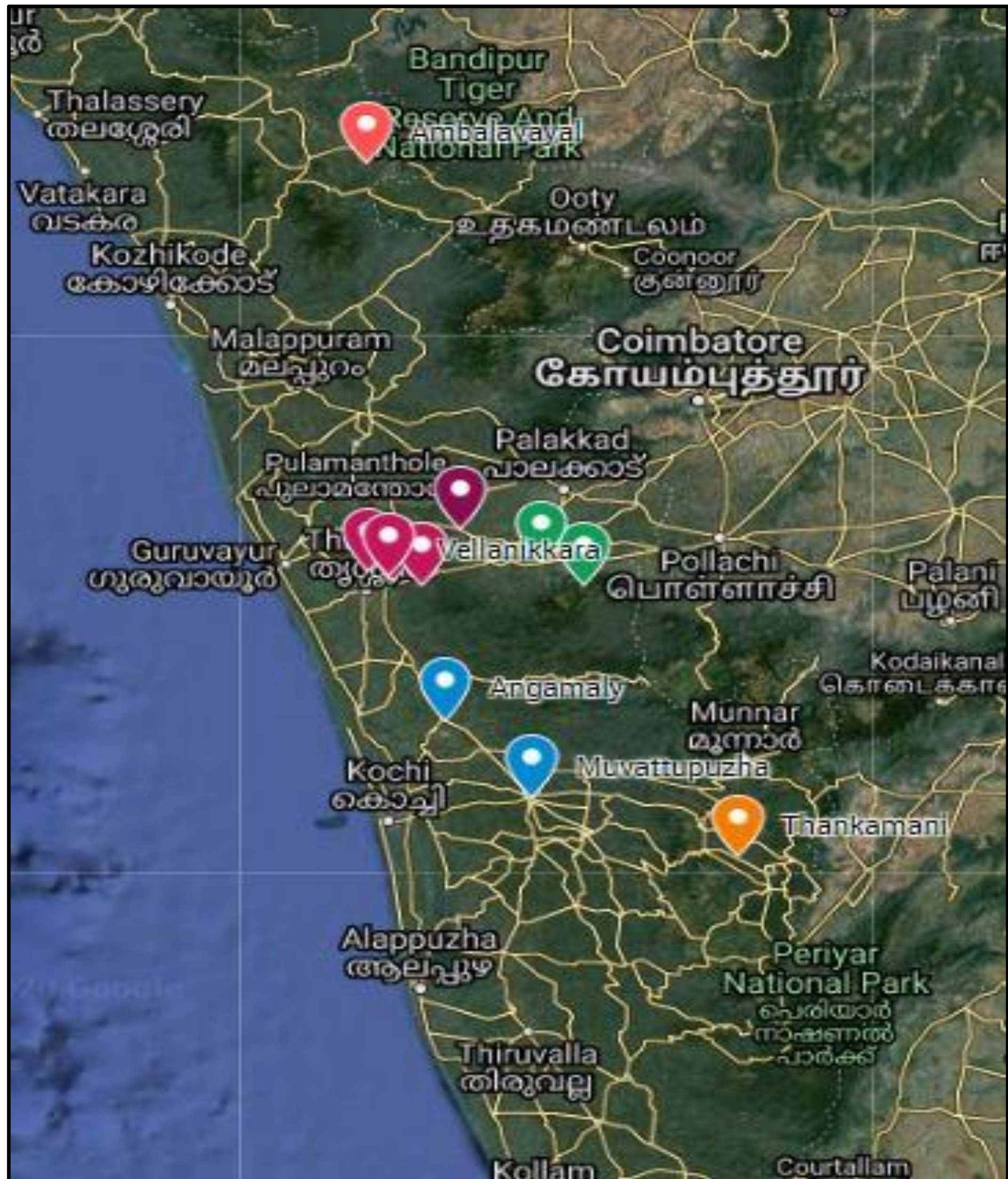


Table 3.2: Cataloguing of diseased samples from different location

Plate 1: Locations of purposive sampling survey

- Thrissur – Vellanikkara, Kannara, Pazhayannur, Madakkathara, Kolazhy
- Ernakulam – Angamaly, Muvattupuzha
- Palakkad – Nelliyambathy, Nenmara
- Wayanad – Ambalavayal
- Idukki - Thankamani

Sl. No.	District	Location / Abbreviation	Symptoms observed/abbreviation	Designated code for the symptoms
1.	Thrissur	Vellanikkara (VKA)	Leaf spot (LS)	VKALS
			Leaf malformation (LM)	VKALM
		Madakkathara (MDK)	Leaf spot (LS)	MDKLS
			Fruit rot (FR)	MDKFR
		Kannara (KAN)	Wilt (Wt)	KANWt
		Kolazhy (KLY)	Bud blight (BB)	KLYBB
		Pazhayannur (PAZ)	Fruit rot (FR)	PAZFR
2.	Ernakulam	Manjapra (MNP)	Wilt (Wt)	MNPWt
			Leaf spot (LS)	MNPLS
		Muvattupuzha (MVP)	Leaf spot (LS)	MVPLS
			Leaf blight (LB)	MVPLB
			Fruit rot (FR)	MVPFR
3.	Palakkad	Nelliyampathy (NEL)	Leaf spot (LS)	NELLS
			Fruit rots (FR)	NELFR
		Nemmara (NEN)	Leaf spot (LS)	NENLS
			Leaf blight (LB)	NENLB
4.	Wayanad	Ambalavayal (AMB)	Leaf spot (LS)	AMBLS
5.	Idukki	Thankamani (THN)	Leaf spot (LS)	THNLS

Plant parts showing symptoms of fungal diseases like spots, blights, rots and anthracnose, *etc.* were kept in moist chamber for further symptom development and sporulation, if any. From these samples, isolation of fungi was carried out as detailed in section 3.2.1.

In the case of bacterial diseases, *in situ* ooze test was conducted in the field itself to confirm the association of bacterial pathogen and the plant parts showing the infection were brought to laboratory for isolation and further studies.

In the case of viral diseases, the leaves showing typical virus like symptoms *viz.* curling, puckering, vein banding, cupping and malformations were brought to the laboratory in ice boxes and kept at -20°C in deep freezer. Three noded cuttings from the infected plants were planted in polybags and virus culture was maintained under insect proof conditions in the virology net house of Department of Plant Pathology, College of Horticulture, Vellanikkara.

3.1.3 Assessment of disease incidence

During the course of survey, the incidence of various symptoms was recorded from the respective fields. For each type of symptom, per cent disease incidence (PDI) was calculated by counting the infected plants showing similar type of symptoms out of the total number of plants from each field. The PDI was calculated using the formula given by Wheeler (1969).

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

3.1.4 Assessment of disease severity

The percent disease severity (PDS) was assessed for the foliage and flower diseases following the standard score chart of 0 - 4 scale (Duarte *et al.,* 2010) with slight modifications as depicted in Table 3.3.

Table 3.3: Score chart for assessing the disease severity

Grade	Description
0	No symptom
1	</ 25 per cent area infected
2	25.1 - </ 50 per cent area infected
3	50.1 - </ 75 per cent area infected
4	75.1 - </ 100 per cent area infected

In the field, similar type of symptoms was scored by taking representative samples using the standard score chart. Later, the per cent disease severity was calculated for each type of symptoms, separately using the formula suggested by Wheeler (1969).

$$PDS = \frac{\text{Sum of all ratings x 100}}{\text{Total number of samples assessed x maximum disease grade}}$$

3.2 ISOLATION OF PATHOGENS

The different parts of plants *viz.* leaves, fruits, flowers and roots showing typical symptoms taken from the fields were brought to the laboratory and washed thoroughly using tap water in order to remove extraneous dust and saprophytic organisms adhering to it.

3.2.1 Isolation of fungal pathogens

The fungal pathogens were isolated following tissue segmentation method (Rangaswamy, 1958). Infected plant tissue along with adjacent healthy portion from the advancing region were cut into small bits of 1.0 to 1.5 cm size using sterile blades. The bits were then surface sterilized using sodium hypochlorite (1%) solution for one minute and subsequently washed in three changes of sterile water in order to remove traces of sodium hypochlorite. A sterile blotting paper was used to remove excess moisture from the sample bit. The bits were then placed aseptically on to sterile Petri plates plated with solidified potato dextrose agar (PDA) medium (Appendix I). Three bits were placed in each plate. Antibiotics were added in order to prevent bacterial

contamination. The plates after inoculation were incubated at room temperature (26 ± 2°C) for five days. The growth of fungi was monitored daily and the hyphal tips were transferred aseptically to solidified PDA in Petri plates and the isolates were then purified by single spore isolation or single hyphal tip method to PDA test tube slants for the maintenance of fungal cultures for further studies. The slants were maintained under refrigerated condition at 4°C.

3.2.2 Isolation of bacterial pathogens

The bacteria were isolated by collecting the ooze in sterile water from the infected tissue and it was then streaked onto nutrient agar medium (Appendix I) plated in sterile Petri dishes by quadrant streak method. The plates were then incubated at room temperature (26 ± 2°C) in an inverted position. After 48 h typical single colonies were selected and sub cultured in nutrient agar for purification. The pure cultures of bacteria were stored in sterile water at room temperature.

3.3 PATHOGENICITY

The pathogenicity of isolates was established following Koch's postulates. Typical symptoms produced by each isolate under artificial conditions were studied by inoculating the pathogen artificially, by placing mycelial discs on different parts of healthy plants where the symptoms expressed under natural conditions.

3.3.1 Pathogenicity of fungal isolates

Mycelial Bit Inoculation Method (MBIM) described by Rocha *et al.* (1998) was followed in order to establish pathogenicity of each fungal pathogen for inoculation on detached leaves, fruits and also on live plants. From fresh cultures of the pathogenic fungi, mycelial plugs of 8.0 mm were cut using cork borer and inoculated on the respective plant parts in an inverted position on adaxial side of the leaf and sides of fruits following pin prick method (Jadesha *et al.*, 2012). Moist cotton swabs were placed over the inoculation bits to maintain the humidity. The inoculated plant parts were placed at ambient room temperature (25ºC) and observations were recorded daily till the development of symptoms. After the appearance of symptoms, the fungal pathogen was reisolated on PDA to confirm the association of pathogen.

3.3.2 Pathogenicity of bacterial isolates

Bacteria grown in freshly prepared nutrient broth were utilized for establishing the pathogenicity. The optical density of nutrient broth was adjusted to OD_{600} = 0.3 nm with a concentration of 1 x 10^8. cfu/ml. Three to four weeks old passionfruit seedlings were used for the pathogenicity studies. Prior to inoculation, the plants were kept in moist chamber for 48 h after watering in order to get maximum infection and disease development. Bacteria culture (24 h old) were inoculated on to the seedlings by drenching after root clipping. The inoculated plant parts were placed at ambient room temperature (25ºC) and observations were tabulated daily for checking the appearance of symptoms. After the appearance of symptoms, ooze test was carried out with the infected seedlings for the confirmation of the infection and the bacterial suspension collected from the plants was plated on nutrient medium to confirm the presence of bacteria.

3.4 SYMPTOMATOLOGY

Detailed studies on symptoms produced by different pathogens were carried out under natural as well as on artificial inoculation.

3.4.1 Under natural conditions

Typical symptoms produced by fungal, bacterial and viral pathogens on different plant parts under natural conditions were observed and documented during the purposive sampling survey conducted in various fields.

3.4.2 Under artificial conditions

In order to study the symptoms produced by the pathogens under artificial conditions, each isolate was inoculated on to the respective plant parts of passion fruit following standard protocol mentioned in 3.3.1 and 3.3.2 Various observations like colour and size of lesions and the time required for characteristic symptom development were noted.

3.5 CHARACTERIZATION AND IDENTIFICATION OF PATHOGENS

3.5.1 Characterization of fungal isolates

The pathogenic fungi were characterized and identified up to generic level based on cultural and morphological characters and selected isolate of each pathogen was subjected to molecular characterization for the identification of the fungi at species level.

3.5.1.1 Cultural characterisation

Cultural characteristics of fungi *viz.* colour, texture, growth rate, pattern of growth, sporulations, pigmentation, colour on the back side of Petri plates and presence of fruiting bodies were recorded. Visual observations on these characters were made daily and recorded. For the study, different isolates were grown on solidified PDA medium in Petri dishes which were incubated at 26 ± 2°C and observations were recorded after attaining full growth.

3.5.1.2 Morphological characterisation

The various microscopic observations like colour of hyphae, branching pattern, septation, presence of conidia, conidial septation, types, shape and size of spores, length and breadth of spores and presence of sexual structures were made. Slide culture technique of Riddle (1950) followed by the microscopic observations were used for studying these morphological characters.

3.5.1.3 Identification of fungal pathogens

The cultural and morphological characters of the fungal isolates were compared with CMI descriptions of plant pathogenic fungi and bacteria (CMI, 1964) and the pathogens were identified.

3.5.2 Characterization of bacterial pathogens

The bacterial pathogens isolated from the passion fruit were characterized based on cultural, morphological, biochemical and molecular characterization.

3.5.2.1 Cultural characterization

The colour and texture of colonies, pigmentation and mucoidal nature of the bacterial colonies were observed on different media like Nutrient Agar (NA) and Tetrazolium Chloride medium (TZC) (Hugh and Leifson, 1953) [Appendix II]. The visual observation on these characteristics were made after inoculating the bacterial isolates on to solidified medium taken in sterile Petri plates and incubated for 48 h in inverted position.

3.5.2.2 Morphological characterization

The size and shape of the bacterial isolates were included under the morphological characters. These observations were made by Gram staining and by Electron Microscopy. Scanning electron microscopic observation was done at Central Instrumentation Laboratory, College of Veterinary and Animal Science, Mannuthy. The protocol used for the Scanning electron microscopy was a slight modification of the protocol described by Li *et al*. (2016).

Protocol

A loopful of bacterial cells suspended in 1.5 ml of sterile water was centrifuged at 6000 rpm for 5 min. After centrifugation, the supernatant was removed and 1.5 ml phosphate buffer saline (PBS) having a pH of seven was added. The contents were again centrifuged at 6000 rpm for 5 min. and the supernatant was removed. After the removal of supernatant, 1.5 ml of glutaraldehyde (2.5%) was added and the mixture was incubated at 37°C for 1.5 h with intermittent vortexing. After the incubation, the content was washed three times with PBS followed by serial dehydration with ethanol (30%, 70%, 80% and 90%) for 10 min. After this, 100 per cent ethanol (1.5 ml) was added and incubated for 1 h. The contents were centrifuged for 6000 rpm for 5 min. after the incubation period. The supernatant was discarded and the sample was air dried. For taking scanning electron micrographs, the sample was smeared on a stub and sputter coated with gold and loaded into scanning electron microscope. The optimum working distance of the electron beam for taking high resolution images of bacterial cells were standardized by trial and error method.

3.5.2.3 Biochemical characterisation

The biochemical tests were conducted in order to identify the bacteria. The different biochemical tests conducted were amylose production test, hydrolysis of gelatin, casein hydrolysis, urease test, indole production, citrate test and oxidase test. The composition of different media used for various biochemical tests are given in Appendix III.

3.5.2.3.a Amylase production test / Starch hydrolysis

Starch agar medium was prepared and autoclaved. Sterilized starch agar medium was then poured into sterile Petri plates and allowed for solidification. Bacterial isolates were streaked at the centre of plate and incubated for 48h at 37°C. After the incubation, the surface of plate was flooded with iodine solution using dropper for 30 seconds and excess iodine was poured off from the plate.

3.5.2.3.b Hydrolysis of gelatin

Nutrient gelatin agar medium was prepared and dispensed in test tubes and autoclaved for preparation of nutrient gelatin deep tubes. Bacterial isolates were stab inoculated in agar deep tubes prepared. An uninoculated agar deep tube was kept as a control and the tubes were incubated at 37°C for 4-7 days. After the incubation period, the tubes were kept under refrigerated condition at 4°C for 15 min.

3.5.2.3.c Casein hydrolysis

Skim milk agar medium was prepared and autoclaved. The media was poured into the Petri plate and allowed for solidification. The bacterial isolates were then inoculated on to the medium by single streak and one control was maintained.

3.5.2.3.d Urease test

Urea agar medium was prepared and sterilized. After cooling the medium, 1g glucose and 6ml of phenol red (0.2%) were added and it is then steamed for 1h. An aqueous solution of urea (20%) was mixed to the medium and the resulting medium was sterilized by filtration. Slants were prepared using this media and bacterial cultures were inoculated and kept for incubation at 37°C for 24 - 48 h.

3.5.2.3.e Indole production test

Fresh tryptone broth (1%) was prepared and sterilized by autoclaving. Each of the bacterial isolate was inoculated on to the medium and one control was maintained without inoculation. After inoculation, the inoculated media were kept for incubation for 48h at 35°C. After that, 1ml of Kovac's reagent was added to inoculated tubes and was gently shaken after 10 – 15 min.

3.5.2.3.e Citrate fermentation test

Simmond's citrate agar slants were prepared and inoculated with bacterial isolates by streaking. An un inoculated slant was maintained as control. The tubes were kept at room temperature and observed for colour change.

3.5.2.3.f Oxidase test

The test was performed by smearing a loopful of the bacterial culture on the oxidase disc impregnated with the substrate, 1 per cent tetramethyl-P-phenyline diamine dihydrochloride.

3.5.2.3 Identification of bacterial pathogen

The cultural and morphological characters of the bacterial isolates were compared with CMI descriptions of plant pathogenic fungi and bacteria (CMI, 1964) and the biochemical characters were compared with the available literature and bacteria were identified (Das and Chattopadhyay (1955); Sharma and Singh (2019); Singh *et al.* (2015); Shekhawat *et al.* (2000); Seleim *et al.* (2014); Sharma and Singh (2019) and Hossain *et al.* (2007)

3.5.2.5 *Identification of races*

The race identification of the identified bacterial pathogens was done by artificial inoculation on differential hosts as suggested by Buddenhagen *et al.* (1962), Aragaki and Quinon (1965) and He *et al.* (1983).

Table 3.4: Differential hosts for identification of races

Races	Differential hosts	Reference
Race 1	Solanaceous vegetables	Buddenhagen *et al.* (1962)
Race 2	Triploid banana	
Race 3	Potato and tomato	
Race 4	Ginger	Aragaki and Quinon (1965)
Race 5	Mulberry	He *et al.* (1983)

The reaction of specific host differentials to the bacterial isolates was tested by raising the differential host in sterilized potting mixture and artificially inoculating those with the bacterial isolates in the method described in section 3.3.2.

3.5.3 Characterisation of virus

Viral like symptoms collected from the surveyed locations were characterised by biological and morphological characters.

3.5.3.1 Biological characterisation

Transmission studies like sap transmission and graft transmission were carried out in order to characterise the virus biologically to identify whether the virus can mechanically transmit at the time of pruning and during propagation.

Mechanical transmission

Passion fruit vines infected with leaf and fruit malformation were used as source of virus inoculum. Seedlings of cowpea were raised in polybags under insect proof conditions. Leaf showing typical symptoms of the virus was taken and fine homogenate was prepared by grinding the symptomatic sample with ice cold 0.1 M phosphate buffer (pH 7) [Appendix IV] using a precooled mortar and pestle. Homogenate was filtered through cotton and the inoculum was maintained in cold condition until inoculation. Cowpea plants at cotyledon leaf stage were used as the test plant and the leaves

uniformly dusted with fine powder of carborundum (600 mesh) prior to inoculation. The filtered sap was rubbed on the leaf surface carefully using cotton in one direction from petiole towards apex of leaves. In order to maintain control, carborundum along with phosphate buffer was applied. After five minutes, the surface of inoculated leaves was washed with distilled water to remove the extraneous particles and excess inoculum. The inoculated leaves were properly labelled and kept for incubation at room temperature under insect proof conditions and observed daily for appearance of symptom.

Graft transmission

Cleft grafting was performed using scions showing typical symptoms taken from infected plants on six months old root stock. The grafts were kept in moist chamber and observations were taken daily to examine graft union and per cent transmission of virus.

3.5.3.2 Morphological characterisation

In order to study the morphology of viral particles associated with the symptoms, transmission electron microscopic studies were conducted using symptomatic leaves collected from infected plants and were sent to Advance Centre for Plant Virology, IARI, New Delhi.

Leaf dip method was the protocol followed for electron microscopy. Infected leaf (1 cm^2) was taken and crushed using 0.1 M phosphate buffer (pH 7) containing one per cent nicotine sulphate on a clean glass slide. A drop of extract was placed on the carbon coated grid of the electron microscope and allowed to stay for 2 minutes. It was followed by washing the grid with ten drops of distilled water and staining with 2 per cent uranyl acetate. Then the excess stain was removed by touching the edge of the grid with a piece of filter paper and it was examined under transmission electron microscope. The electron micrographs of the virus particles were taken on a plate film. The size and shape of the viral particles were documented.

3.6 MOLECULAR CHARACTERIZATION

Fungal, bacterial and viral pathogens isolated from the diseased samples of passion fruit collected during the purposive sampling survey conducted in five districts

of the state were subjected to molecular characterization to identify the organism at the species level. In the case of fungal and bacterial pathogens, molecular characterization was carried out by isolation of genomic DNA and amplification of a specific region *viz.* ITS (Internal Transcribed Spacer) region and 16S rRNA of the genome of fungal and bacterial pathogens respectively. The molecular diagnosis of virus associated with passion fruit was carried out by the isolation of total RNA from the infected sample followed by cDNA synthesis and amplification of region specific to the cylindrical inclusions (CI) in the viral genome.

3.6.1 Molecular characterization of fungal pathogens

All the fungal pathogens isolated from the samples collected during the purposive sampling survey were characterized up to the species level by the isolation of fungal DNA followed by sequencing of the ITS – rDNA region.

3.6.1.1 DNA isolation protocol (CTAB method)

1. 100- 150 mg of clean fungal mycelium was grinded in a prechilled mortar and pestle with a pinch of PVP and 50 µl of β-mercapto ethanol in the presence of liquid nitrogen.
2. The homogenized sample was transferred into an autoclaved 2 ml centrifuge tube and 1 ml of pre warmed extraction buffer [Appendix V] was added.
3. The contents were mixed well and the mixture was incubated at 65°C for one hour.
4. To this mixture, equal volume of chloroform- isoamyl alcohol (24:1) was added and mixed by inversion to emulsify.
5. The mixture was then centrifuged at 10000 rpm for 15 min. at 4°C.
6. After centrifugation, the top aqueous layer was transferred to a clean tube and to this, $1/10^{th}$ volume of 10 per cent CTAB solution and equal volume of chloroform-isoamyl alcohol (24:1) was added and mixed gently by inversion.
7. The content was then again centrifuged at 10000 rpm for 15 min. at 4°C.
8. The aqueous phase was then transferred to a clean tube and 0.6 volume of chilled isopropanol was added and mixed by quick and gentle inversion till the DNA precipitates. The tube was kept at -20°C for half an hour for complete precipitation.

9. After complete precipitation, it was then centrifuged at 10000 rpm for 15 minutes at 4°C and the supernatant was poured off.
10. The DNA pellet was washed with 70 per cent ethanol followed by 100 per cent ethanol by centrifugation for 3 min. at 10000 rpm and decanting.
11. The pellet was air dried and dissolved in 50 µl of TE buffer or sterile water and stored at -20°C.

3.6.1.2 Agarose gel electrophoresis

The purity of isolated DNA samples was determined with agarose gel electrophoresis using 1X TAE buffer in BIORAD gel electrophoresis unit. Agarose gel (0.8%) was prepared in 1 X TAE buffer containing 0.5 µg ml^{-1} of ethidium bromide. An aliquot of DNA samples (5µL) of the fungal isolates mixed with 1 µl of gel loading dye was loaded in each of the wells along with 1 kB ladder to determine the size of DNA. Electrophoresis was carried out at 70 V till the dye moved one by third of the gel. The gel was visualized and the image was documented using Univech Gel Documentation System.

3.6.1.3 Spectrophotometric analysis

The quality and quantity of the isolated DNA was determined by recording the concentration and absorbance value of the DNA samples using Nanodrop spectrometer (IMPLEN Photometer). The instrument was calibrated to blank (zero absorbance) using 1µl sterile water. The absorbance of the sample was observed at 260 nm and 280 nm and the purity was expressed as their ratio.

3.6.1.4 Standardization of PCR

The conditions for amplification of ITS region of the fungal genome was standardized by trial and error method.

Standardization of PCR conditions for reported primers:

Primers specific to the ITS region were used in the PCR for characterization of fungal pathogen isolates collected from different surveyed locations and the details regarding the primers were given in Table 3.4.

PCR was carried out in a Thermo Cycler (Eppendof Mastercycler) using the reported primers. Gradient PCR was done in order to standardize the annealing temperature at using the Vertiflex program in the Thermo Cycler.

The components of the PCR reaction mixture used for PCR is described in Table 3.5 and those were mixed in a mini spinner and proceeded with the reaction.

Table 3.5: **Details of primer used for molecular characterisation of fungal isolates**

Target	Primer Name	Direction	Sequence (5’⟶3’)
ITS	ITS- 1F	Forward	TCCGTAGGTGAACCTGCGG
	ITS- 4R	Reverse	TCCTCCGCTTATTGATATGC

Table 3.6: **Components of PCR reaction**

Sl. No.	Component	Quantity (µl)
1.	Template DNA (50 ng/µl)	2
2.	10X PCR buffer (with 1.5Mm $MgCl_2$)	3
3.	dNTP mixture (2.5mM each)	3
4.	Forward Primer	1
5.	Reverse Primer	1
6.	*Taq* polymerase	1
7.	Water	19
	Total reaction mixture	30

PCR amplification profile:

After the annealing temperature was standardized, reaction was carried out as follows in a Thermo Cycler (Eppendof Mastercycler)

Initial denaturation - 94°C – 4.00 min

Step			
Denaturation	- 94°C – 1.00 min	}	40 cycles
Annealing	- 48.00 - 54°C – 1.00 min	}	
Extension	- 72°C – 1.5 min	}	

Final extension - 72°C – 5.00 min

3.6.1.5 Gel documentation of PCR products

Once the reaction was over, the PCR products were run on 1.2 per cent agarose gel prepared on 1 X TAE buffer containing 0.5 µg/ ml of ethidium bromide. Sample (5 µl) was loaded on to the well along with 1 µl of gel loading dye. 100 base pair ladder (Thermoscientific, USA) was also run along with the sample in order to find the size of amplicon. Electrophoresis was done at 75 V power supply with 1X TAE buffer as the electrophoresis buffer till the dye moved 8 cm from the well. The amplified product was visualized and the image was documented using Univech Cambridge gel documentation system.

3.6.1.6 Sequencing

The PCR products amplified using ITS primers were sent to Agri Genome, Kochi for purification and sequencing. The sequence information of ITS region retrieved from Agri Genome was analyzed further using bioinformatics tools for the characterization of fungal isolates.

***3.6.1.7 In silico* analysis of sequences**

The sequences of ITS regions corresponding to each pathogen were further analyzed. Homology search was done using the bioinformatics tool, BLAST in which the sequences were compared with the sequences available in the NCBI database (https://blast.ncbi.nlm.nih.gov./Blast.cgi) to get the best aligned sequences.

3.6.1.8 Phylogenetic analysis

Phylogenetic analysis of fungal isolates belonging to the same species and belonging to same genus was carried out in MEGA 7.0 software by constructing the phylogenetic tree using Neighbour –Joining bootstrap method (Saitou and Nei, 1987).

3.6.1.9 DNA Barcoding

Multiple sequence alignment of the sequences of fungal isolates belonging to the same genus (collected during the survey) and sequences of the same collected from the NCBI database was created using ClustalW tool provided by MEGA-X software. The aligned sequences of ITS region were manually analyzed for the presence of barcode gaps. The region with identical nucleotides within the species and differ between species were identified as barcodes specific to that particular species.

3.6.2 Molecular characterization of bacterial pathogens

All the bacterial pathogens isolated from the diseased samples collected during the purposive sampling survey were characterized up to the species level by the isolation of bacterial DNA followed by the 16S rRNA sequencing.

3.6.2.1 Genomic DNA Isolation from bacteria

Genomic DNA of bacteria was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions.

A part of culture was taken in a microcentrifuge tube. 180 µl of T1 buffer and 25 µl of proteinase K was added and incubated at 56 ^{o}C in a water bath until it was completely lysed. After lysis, 5 µl of RNase A (100 mg/ml) was added and incubated at room temperature for 5 min. 200 µl of B3 buffer was added and incubated at 70°C for 10 minutes. 210 µl of 100 per cent ethanol was added and mixed thoroughly by vortexing. The mixture was pipetted into NucleoSpin® Tissue column placed in a 2 ml collection tube and centrifuged at 11000 x g for 1 minute. The NucleoSpin® Tissue column was transferred to a new 2 ml tube and washed with 500 µl of BW buffer. Wash step was repeated using 600 µl of B5 buffer. After washing the NucleoSpin® Tissue column was placed in a clean 1.5 ml tube and DNA was eluted out using 50 µl of BE buffer.

3.6.2.2 Agarose gel electrophoresis for DNA quality and quantity check

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25% bromophenol blue, 30% sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8% agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg ml^{-1}

ethidium bromide. Electrophoresis was performed with 0.5X TBE as electrophoresis buffer at 75 V until bromophenol dye front has migrated to the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.5.2.4 *PCR Analysis*

The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). Details regarding composition of PCR master mix, primer used for amplification and PCR amplification profile were given in table 3.7 and 3.8 respectively.

Table 3.7: Composition of PCR master mix

2X Phire Master Mix	5 µl
D/W	4 µl
Forward Primer	0.25 µl
Reverse Primer	0.25 µl
DNA	1 µl

Table 3.8: Details of primers used for molecular characterisation of bacterial isolates

Target	Primer Name	Direction	Sequence (5’ → 3’)
16S Rrna	16S-RS-F	Forward	CAGGCCTAACACATGCAAGTC
	16S-RS-R	Reverse	GGGCGGWGTGTACAAGGC

PCR amplification profile:

95 °C	-	5.00 min	
95 °C	-	30 sec	35 cycles
60 °C	-	40 sec	35 cycles
72 °C	-	60 sec	35 cycles
72 °C	-	7.00 min	

4 ^{o}C - ∞

3.6.2.4 Agarose Gel electrophoresis of PCR products

The PCR products were checked in 1.2% agarose gels prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide. 1 µl of 6X loading dye was mixed with 4 µl of PCR products and was loaded and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The molecular standard used was a 2-log DNA ladder (NEB). The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad).

3.6.2.5 In silico analysis of sequences

The sequences of 16S rRNA region corresponding to the bacterial pathogen was further analyzed. Homology search was done using the bioinformatics tool, BLASTn in which the sequences were compared with the sequences available in the NCBI database (https://blast.ncbi.nlm.nih.gov./Blast.cgi) to get the best aligned sequences.

3.5.3 Molecular characterisation of Virus

The experiments associated with molecular characterisation on the virus were carried out at Advanced Centre for Plant disease diagnosis, Department of Plant Pathology, College of Horticulture, Vellanikkara. The leaves with typical symptoms of viral infection were collected from the passion fruit orchards during early morning hours and brought to the laboratory in ice box for RNA isolation.

3.6.3.1 Isolation of RNA

Total RNA from the infected leaf sample was isolated using TRIzol reagent (Sigma Aldrich) method. All the glass wares and micropipette tips used for RNA isolation were treated with 0.1 per cent Diethyl Pyrocarbonate (DEPC) overnight prior to the isolation and double autoclaved at 121° C temperature and 15 lbs pressure. The infected leaf sample collected from the field was wiped with RNase ZAP to deactivate the RNases present. 100 mg of the leaf sample was ground to fine powder using liquid nitrogen with pestle and mortar along with a pinch of PVP and 50 µl β- mercapto ethanol. One ml of TRIzol reagent was added to homogenize the powdered tissue. It

was then transferred to 2 ml RNase free microcentrifuge tubes and incubated till the homogenate developed a brown colour. This was then centrifuged at 13,000 rpm for 10 minutes at 4°C. The supernatant was then transferred into fresh 2ml centrifuge tube and to this 200 µl of chloroform was added and the contents were mixed well using a vortex mixer which was then incubated at room temperature for 10 minutes. Separate three phases were formed in the centrifuge tube when centrifuged at 13,000 rpm for 15 min at 4°C. The colourless upper phase from the three layers containing the RNA was carefully pipetted into a fresh 2 ml microcentrifuge tube and an equal amount of isopropanol was added into this which was followed by slow mixing by inversion and incubation for one hour in ice. The content was then centrifuged at 13,000 rpm for 10 min at 4°C. After centrifugation, the supernatant was discarded and the pellet was washed with 75 per cent ethanol by centrifugation at 7500 rpm for 10 min at 4°C. The pellet formed was air dried and suspended in 20 µl of autoclaved DEPC water. The same method was followed to isolate RNA from leaf samples collected from healthy plants which served as the control.

3.6.3.2 Gel documentation

The purity of RNA was checked using Agarose gel (1%) in 1X TAE buffer. An aliquot of RNA sample (5µl) was mixed with 1µl loading dye and loaded into the wells of the gel. Electrophoresis was carried out at 50 V till the dye moved 5 cm from the well (in BIORAD gel electrophoresis unit). The gel was then visualized under uv light and the image was documented in Univech Cambridge gel documentation system.

3.6.3.3 Spectrophotometric analysis

The quantity and quality of the RNA was determined by recording the concentration and the absorbance value of the samples using IMPLEN Spectrophotometer. The spectrophotometer was calibrated to zero absorbance with one µl of sterile DEPC water. The concentration of RNA in the sample and absorbance at 260 and 280 nm wavelength was recorded. The purity was indicated by the ratio $A_{260/280}$.

3.6.3.4 Synthesis of First Strand Complementary DNA (cDNA)

Complementary DNA was synthesised from the isolated RNA using Revert Aid First Strand cDNA synthesis kit (Catalogue No: K1621, Thermoscientific) as per

manufacturer's instructions. Different components in the reaction mixture was given in table 3.9. These components were added into sterile nuclease free polypropylene PCR tubes on ice in the sequential order given in table3.9. The contents were mixed gently and incubated at 42°C for 60 min in PCR machine (Eppendof MasterCycler) followed by heating at 70°C for 5 min for termination.

Table 3.9: Reagents used for cDNA symthesis

Sl. No.	Name of the component	Volume (µl)
1.	RNA template	2
2.	Oligo $(dT)_{18}$ primer	1
3.	Water (nuclease free)	9
4.	5X reaction buffer	4
5.	Ribolock RNase inhibitor 20 U/µl	1
6.	10 Mm dNTP mix	2
7.	Revert Aid M-MuL V RT (200u/ µl)	1
	Total volume	20

3.6.3.5 Standardisation of Polymerase Chain Reaction (PCR)

Molecular characterisation of viral pathogen collected from infected leaf samples of passion fruit was carried out by the amplification of CI region of the viral genome using primers reported by Ha *et al.* (2008).

Primer used: PCR was carried out using degenerate primer specific to the Cylindrical Inclusion (CI) protein gene and the sequence of forward and reverse primer was given in Table 3.10.

Table 3.10: Details of reported primers used for amplification

Primer	Sequence (5'-3')	Length	Tm value (°C)
CIFor	GGIVVIGTIGGIWSIGGIAARTCIAC	26	63.3
CIRev	ACICCRTTYTCDATDATRTTIGTIGC	26	61.0

PCR was carried out in a thermocycler (Eppendof MasterCycler) with the above mentioned degenerate primers.

Standardisation of cDNA dilution: The reaction was carried out at 4 different dilutions of cDNA *viz.* 1:1, 1:0.5, 1:0.25 and 1:0.1 in order to standardize the optimum dilution of cDNA template for PCR amplification.

Standardisation of annealing temperature: The temperature for annealing was standardized using the Vertiflex program in Eppendof Master Cycler with a gradient of temperature ranging from 40°C to 50°C. Suitable annealing temperature was selected based on the quality of DNA band obtained during gel documentation.

The components of the reaction mixture used for PCR was given Table 3.11 and after addition of each of the components, these were mixed gently in a mini spinner and the PCR was carried out.

Table 3.11: Components of reaction mixture for PCR

Sl. No.	Components	Quantity (µl)
1.	Template cDNA	2
2.	10X PCR buffer (with 1.5mM $MgCl_2$)	3
3.	dNTP mixture (2.5mM each)	3
4.	Forward primer	1
5.	Reverse primer	1
6.	*Taq* polymerase	1
7.	Water	19
	Total reaction mixture	30

PCR amplification profile:

After the standardization of annealing temperature and cDNA, RT-PCR was carried out as follows

Initial denaturation - 95°C – 2.00 min

Denaturation	- 95°C – 40 sec	30 cycles
Annealing	- 40.6°C – 55 sec	
Extension	- 72°C – 1.0 min	
Final extension	- 72°C – 5.00 min	

Reaction with healthy samples was served as control to confirm the presence of virus in them.

3.6.3.6 Gel documentation of PCR products

The PCR products were analyzed by gel electrophoresis using 1.2 per cent agarose gel in 1X TAE buffer having 0.5 µg/ ml ethidium bromide. About 5µl of the PCR products was mixed with 1 µl of tracking dye and loaded on the well. 100 bp ladder was also loaded in one well to compare the size of PCR products and electrophoresis was done with 80 V power supply with 1X TAE buffer as the electrophoresis buffer till the dye moved 8 cm from the well. The amplified product was visualized and the image was documented using UNIVEC Gel documentation system.

3.6.3.7 Molecular diagnosis

The size of the band of amplified product were compared with that of the expected product size to confirm the association of virus with the symptoms.

3.7 CORRELATION OF WEATHER PARAMETERS WITH DISEASE SEVERITY

Weather parameters like maximum and minimum temperature, relative humidity and rainfall prevailing in different locations during the period of survey were collected from the official site of Marksim DSSAT weather file generator (http://gismap.ciat.cgiar.org/MarkSimGCM/) for respective locations of purposive sampling survey in Thrissur, Ernakulam, Palakkad, Wayanad and Idukki districts. The data collected was used to correlate with the severity of fungal diseases prevailing in each location.

3.8 *In vitro* EVALUATION OF FUNGICIDES AND BIOCONTROL AGENTS AGAINST MAJOR FUNGAL PATHOGENS.

3.8.1 *In vitro* evaluation of fungicides against major fungal pathogens.

In vitro evaluation of the following fungicides against major fungal pathogens was carried out by poison food technique (Zentmeyer, 1955) at three different doses *viz.* lower, recommended and higher doses (Table 3.12). In the case of Bordeaux mixture only one dose *i.e.* 1 per cent was done. For this chemicals were mixed separately in 100 ml sterilized PDA media and poured into sterilized Petri plates @ 20 ml/plate. After this eight mm mycelial disc of pathogens were placed at the center of poisoned media. Plates without fungicide served as control.

Completely Randomized Block Design was performed with 22 treatments and a replication of three each.

Observations were recorded till the control plates attained full growth of the pathogen. Per cent inhibition of the pathogen with the fungicide was calculated using the formula given by Vincent (1927).

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

C – Growth of the pathogen in the control

T – Growth of the pathogen in treatment

3.8.2 *In vitro* evaluation of bio control agents against major fungal pathogens.

Biocontrol agents *viz. Trichoderma* sp. (KAU reference culture) and *Pseudomonas fluorescens* (KAU reference culture) were tested by dual culture technique (Johnson and Curl, 1972) for the control of the major fungal pathogens of passion fruit collected during the survey.

Table 3.12: Fungicides used and its doses

Sl. No.	Fungicide used	Concentration (Per cent)
1.	Copper hydroxide	0.05, 0.1, 0.2
2.	Hexaconazole	0.1, 0.2, 0.3
3.	Propineb	0.15, 0.25, 0.35
4.	Difenoconazole	0.025, 0.05, 0.075
5.	Carbendazim 12% + mancozeb 64%	0.1, 0.2, 0.3
6.	Cymoxanil8% + mancozeb 64%	0.1, 0.25, 0.5
7.	Azoxystrobin	0.005, 0.01, 0.015
8.	Bordeaux mixture	1.0

3.8.2.1 In vitro evaluation of Trichoderma sp. against major fungal pathogen

For the evaluation of *Trichoderma* sp., sterilized Petri plate containing PDA medium were inoculated with 8 mm mycelial disc of five days old culture of the pathogen at 2 cm from the periphery. After 48 h of incubation, 8 mm disc of five days old culture of *Trichoderma* sp. was placed at the opposite end in the same plate at 2 cm distance from the periphery. Monoculture of the pathogen served as control and three replications were kept and observation were made till the pathogen completed the growth in control.

The types of interaction between the pathogen and fungal antagonist (*Trichoderma* sp.) were recorded using the key developed by Webber and Hedger (1986). It consists of the following types of interaction.

1. Intermingling of 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

3.8.2.2 In vitro evaluation of Pseudomonas fluorescens against major fungal pathogen

The inhibition of major fungal pathogen by the bacterial antagonist, *Pseudomonas fluorescens* was recorded by inoculating the mycelial disc (8 mm) of five days old culture of the pathogen at the centre of Petri plate and the culture of *Pseudomonas fluorescens* was streaked on either side of the pathogen at 2 cm from the periphery of the dish. The pathogen grown as Monoculture served as the control and three replicates were kept for observation. Observation were made till the pathogen completed the growth in control.

The per cent inhibition of the growth of pathogen by the control bio control agents was calculated by the formula given by Vincent (1927).

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

C – Growth of the pathogen in the control

T – Growth of the pathogen in treatment

3.8.3 Statistical analysis

Analysis of the data generated during the experiments was carried out using web agri-stat package (WASP 2.0). Transformation of the data was performed as required. Multiple comparison of the treatment means was done using critical difference method.

Results

4. RESULTS

The study on “Characterization and documentation of diseases of passion fruit (*Passiflora edulis* Sims.) was undertaken with an objective to identify and document the diseases affecting passion fruit grown in the state. The investigation was carried out in the Department of Plant Pathology, College of Horticulture, Vellanikkara during 2018-2020. The results obtained during the course of research are detailed below.

4.1 SURVEY ON THE OCCURRENCE OF DISEASES

Purposive sampling surveys were conducted in major passion fruit growing tracts of the state during 2018-2020 for assessing various diseases affecting passion fruit. The passion fruit orchards from five districts *viz.* Thrissur, Ernakulam, Palakkad, Wayanad and Idukki were surveyed (Plate 2). Twelve locations from five districts were surveyed for the collection of diseased samples (Table 4.1). The sampling survey was conducted with the objective to study each type of symptoms under natural, field conditions, to assess the incidence and severity of various diseases associated with each type of symptoms observed and to collect infected samples from various plant parts *viz.* leaves, fruits, flowers and roots.

Symptoms observed in different locations are given in Table 4.2. Different types of disease symptoms were recorded like leaf spots, leaf blights, fruit rots, bud blight, wilting and leaf malformation during the survey. Leaf spot was the most prominent disease symptom observed in all locations except Kannara, Kolazhy and Pazhayannur. Leaf blight was another symptom observed and was seen in two locations *viz.* Nemmara and Muvattupuzha. Four different types of fruit rots were collected from Vellanikkara, Nelliyampathy, Muvattupuzha and Pazhayannur in which three of them affected mainly the rind leading to reduction in consumer preference and market value while the other one affected the internal contents of the fruit. Blighting of flower buds were recorded from one of the fields (Kolazhy). Wilting of the productive vines were also observed as a serious problem in Thrissur (Kannara) and Ernakulam (Manjapra) district. Leaf malformation associated with the crop was recorded from Vellanikkara (Thrissur).

Each type of symptom was catalogued by assigning a code (Table 4.2). Further investigations on each type of symptomatic samples were carried out on the basis of the

assigned code until the identity of the pathogen and the disease was confirmed. The stage of the crop in the fields was fruit bearing except in Nemmara and Thankamani where the crop was in vegetative stage and the age of crop ranges from one to three and a half year. Majority of the farmers were cultivating yellow variety of passion fruit. Purple cultivars were observed only in Ambalavayal location of Wayanad district.

In Thrissur district, six fields in five different locations were surveyed *i.e.* Vellanikkara, Madakkathara, Kannara, Kolazhy and Pazhayannur. Seven different types of symptoms which includes leaf spots, fruit rot, bud blight, wilting and leaf and fruit malformation were observed and collected. A leaf spot (VKALS) and leaf malformation (VKALM) were recorded from two fields of Vellanikkara. Another leaf spot (MDKLS) and fruit rot (MDKFR) was collected from the field at Madakkathara. The passion fruit plants in a field at Kannara showed wilting (KANWt) of vines as the main symptom. From Pazhayannur, one fruit rot disease (PAZFR) was collected. Blighting of flower buds (KLYBB)was recorded from the field at Kolazhy.

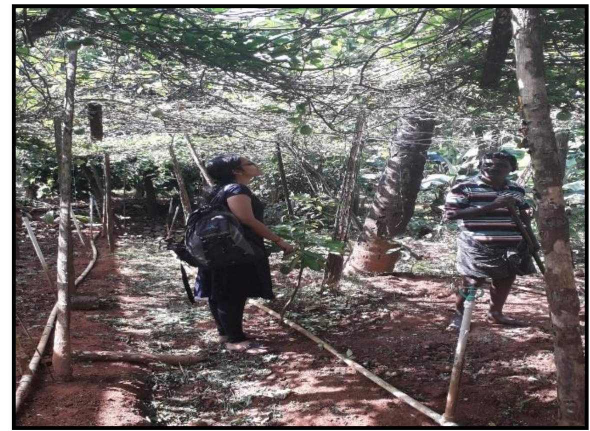
Purposive sampling surveys in Ernakulam district were conducted in two locations *viz.* Manjapra and Muvattupuzha. Five types of disease symptoms *viz.* two leaf spots (MVPLS and MNPLS), one leaf blight (MVPLB), one fruit rot (MVPFR) and one wilt (MNPWt) were recorded. The symptoms MVPLS, MVPLB and MVPFR were collected from Muvattupuzha. The leaf spot (MNPLS) and the wilt (MNPWt) were collected from Manjapra (Angamaly).

The passion fruit orchards in Nelliyampathy and Nemmara were the two locations where purposive sampling survey was conducted in Palakkad district. Two leaf spot (NELLS and NENLS), one leaf blight (NENLB) and one fruit rot (NELFR) were collected from the two locations. The leaf spot (NELLS) and fruit rot (NELFR) were collected from the Nelliyampathy location and the leaf spot (NENLS) and the leaf blight (NENLB) were collected from the location Nemmara.

During the survey, only leaf spots (THNLS and AMBLS) were obtained from the districts Idukki and Wayanad. THNLS was collected from Thankamani of Idukki district and AMBLS was from Ambalavayal of Wayanad district.

THRISSUR **PALAKKAD**

WAYANAD



ERNAKULAM **IDUKKI**

Plate 2: Surveyed locations

4.1.1 Assessment of Per cent disease incidence and Per cent disease severity

During the purposive sampling surveys, different symptoms observed were noted separately and per cent diseases incidence (PDI)/ per cent disease severity (PDS) of each type of symptom was assessed. The details from each location are given in Table 4.3.

Two leaf spots (VKALS and MDKLS), one fruit rot (MDKFR), wilting (KANWt) of vines, bud blight (KLYBB) and one leaf malformation (VKALM) were recorded from Thrissur district. Among the various diseases recorded, maximum per cent disease severity (PDS) of 53.59% was recorded for fruit rot PAZFR with disease incidence of 26.47 per cent. The highest severity among the leaf spot diseases was recorded for VKALS (32.48%) with 18.47 per cent incidence followed by MDKLS (25%) with an incidence of 16.28 per cent. Even though the fruit rot disease (MDKFR) showed an incidence of 39 per cent, the severity was only 13 per cent. Bud blight (KLYBB) was recorded with an incidence of 37.5 per cent. The wilting symptom (KANWt) showed an incidence of 33 per cent and the affected plant was completely destroyed due to infection.

From Ernakulam district, four types of disease symptoms were recorded *viz.* leaf spots (2 nos.), leaf blight (1 no.), fruit rot (1 no.) and wilt (1 no.). Among the different samples collected, the highest per cent disease severity (63.90%) and incidence (78%) was recorded for the leaf blight (MVPLB) followed by leaf spot (MVPLS) with PDS and PDI of 58.73 per cent and 43 per cent respectively. Wilting of the entire vines of passion fruit was found to be more severe in Manjapra of Angamaly region with an incidence of 37.64 per cent in which the entire plant at the fruiting stage was wilted and resulted in total loss to the farmers. The lowest severity (9.25%) was recorded for the leaf spot, MNPLS collected from the same field with an incidence of 14.80 per cent.

From the passion fruit orchards of Nelliyampathy and Nemmara of Palakkad district, two leaf spot samples (NELLS and NENLS), one leaf blight (NENLB) and one fruit rot (NELFR) were observed with per cent disease severity of 38.53, 35.49, 39.66

Table 4.1 Details of survey, locations and crop

Sl. No.	District	Location	Month of survey	Stage of crop	Age of the crop (years)	Variety
1	Thrissur	Vellanikkara (Field 1)	March, 2019	Fruiting stage	2	Yellow
		Vellanikkara (Field 2)	January, 2020	Fruiting stage	1	Yellow
		Madakkathara	August, 2019	Fruiting stage	2	Yellow
		Kannara	September, 2019	Fruiting stage	3	Yellow
		Pazhayannur	October, 2019	Fruiting stage	3	Yellow
		Kolazhy	April, 2019	Fruiting stage	1	Yellow
2	Palakkad	Nelliyampathy	July, 2019	Fruiting stage	3.5	Yellow
		Nemmara	September, 2019	Vegetative	1	Yellow
3	Ernakulam	Manjapra, Angamaly	May, 2019	Fruiting stage	2.5	Yellow
		Muvattupuzha	September, 2019	Fruiting stage	2	Yellow
4	Wayanadu	Ambalavayal	October, 2019	Fruiting stage	2	Purple
5	Idukki	Thankamani	November, 2019	Vegetative	2	Yellow

Table 4.2 Symptoms observed and designated codes

Sl. No.	District	Location	Symptoms	Designated code for the disease
1.	Thrissur	Vellanikkara, (Field 1)	Leaf spot	VKALS
		Vellanikkara (Field 2)	Leaf malformation	VKALM
		Madakkathara	Leaf spot	MDKLS
			Fruit rot	MDKFR
		Kannara	Wilt	KANWt
		Pazhayannur	Fruit rot	PAZFR
		Kolazhy	Bud blight	KLYBB
2.	Ernakulam	Manjapra	Wilt	MNPWt
			Leaf spot	MNPLS
		Muvattupuzha	Leaf spot	MVPLS
			Leaf blight	MVPLB
			Fruit rot	MVPFR
3.	Palakkad	Nelliyampathy	Leaf spot	NELLS
			Fruit rot	NELFR
		Nemmara	Leaf spot	NENLS
			Leaf blight	NENLB
4.	Wayanad	Ambalavayal	Leaf spot	AMBLS
5.	Idukki	Thankamani	Leaf spot	THNLS

and 36.76 respectively. Among these samples collected, NENLB showed highest incidence of 95 per cent followed by NELLS (88.75%), NELFR (78.25%) and NENLS (75%).

From Idukki and Wayanad districts, only leaf spot symptoms were obtained. The two leaf spots; AMBLS (from Wayanad) and THNLS (from Idukki) were observed with a severity of 40.23 per cent and 40.84 respectively. These isolates showed incidence of 48.90 (AMBLS) per cent and 39.92 (THNLS) per cent.

4.2 ISOLATION OF PATHOGENS

During purposive sampling surveys, plant parts showing various disease symptoms *viz.* leaves, fruits, flowers and roots were collected from different locations and brought to the laboratory for further studies. The pathogen associated with each type of symptom was isolated following the protocols explained in the section 3.2 and then maintained by frequent sub culturing on PDA slants.

4.3 PATHOGENICITY OF DIFFERENT ISOLATES

The pathogenicity of 17 microorganisms (fungal and bacterial) isolated from the infected samples of passion fruit collected from different locations was established by artificial inoculation and reisolation under *in vitro* conditions. The isolates were inoculated on healthy plant parts where the symptoms observed naturally under field conditions. The protocols followed for pathogenicity tests are described in section 3.3 and the symptoms appeared after the inoculation of the pathogens are documented in section 4.4.2.

The incubation period *i.e.* the time taken to develop symptoms upon artificial inoculation ranged from two to eight days and the same for each isolate are given in Table 4.4. The reisolation from the inoculated symptomatic region of each isolate yielded the same pathogen, thus proving the Koch's postulates.

4.4. SYMPTOMATOLOGY

Detailed study on symptom development associated with each disease was carried out both under natural conditions and by artificial inoculation of the pathogen. Symptomatology under natural conditions were documented during the survey from

Table 4.3 Incidence and severity of diseases of passion fruit in different locations

Sl. No.	Location	Diseases	PDS (%)	PDI(%)
1.	Vellanikkara (Field 1)	VKALS	18.47	32.48
2.	Vellanikkara (Field 2)	VKALM	75.38	89.94
3.	Madakkathara	MDKLS	16.28	25.00
		MDKFR	13.00	39.00
4.	Kannara	KANWt	-	33.00
5.	Pazhayannur	PAZFR	26.47	53.59
6.	Kolazhy	KLYBB	-	37.50
7.	Nelliyampathy	NELLS	38.53	88.75
		NELFR	36.76	78.25
8.	Nemmara	NENLS	35.49	75.00
		NENLB	39.66	95.00
9.	Manjapra, Angamaly	MNPWt	-	37.64
		MNPLS	9.25	14.80
10.	Muvattupuzha	MVPLS	58.73	43.00
		MVPLB	63.90	78.00
		MVPFR	20.39	45.86
11.	Ambalavayal	AMBLS	40.23	48.90
12.	Thankamani	THNLS	40.84	39.92

Table 4.4: **Isolated pathogens and time taken to develop symptoms on artificial inoculation**

Sl. No.	Isolate	Pathogen	Incubation period (days)	Symptoms on artificial inoculation	Reisolation (+/-)
1.	VKALS	Fungus	2	Black, water soaked, lesions with yellow halo	+
2.	MDKLS	Fungus	4	Brown, water soaked spots with concentric zonation	+
3.	MNPLS	Fungus	2	Irregular, water soaked, black lesions	+
4.	NELLS	Fungus	2	Irregular shaped, black lesions	+
5.	AMBLS	Fungus	3	Minute black spots later coalesced to necrotic patch	+
6.	MVPLS	Fungus	2	Small black spots coalesced to necrotic lesion	+
7.	THNLS	Fungus	3	Black water soaked lesion with a narrow yellow halo	+
8.	NENLS	Fungus	2 – 3	Brown necrotic lesion with yellow halo	+
9.	MVPLB	Fungus	4	Minute creamy white spots coalesced to necrotic patch	+
10.	NENLB	Fungus	3	Minute cream spots coalesced to necrotic patch	+
11.	MDKFR	Fungus	4	Light brown greasy spots with dark brown margin	+
12.	NELFR	Fungus	2	Small, brown, water soaked spots	+
13.	MVPFR	Fungus	3	Dark brown, water soaked, sunken spots	+
14.	PAZFR	Fungus	2	Water soaked, pink spots with white cottony mycelia	+
15.	KLYBB	Fungus	5	Water soaked appearance followed by rotting of buds	+
16.	MNPWt	Bacteria	7-8	Drooping of leaves, fading of green colour and wilting	+
17.	KANWt	Bacteria	8	Wilting and complete withering	+

each field and symptom development on artificial inoculation was recorded by inoculating each pathogen on healthy plant parts.

4.4.1 Symptomatology under natural conditions

An elaborate view of the various symptoms observed on different plant parts of the crop during the survey conducted in five districts of the state was explained in the following paragraphs. This includes the foliage symptoms, fruit rots, bud blight, wilting and leaf malformation symptoms.

4.4.1.1 Foliage symptoms

The foliage diseases observed during the survey includes leaf spots and leaf blights. Eight isolates of leaf spot pathogen (VKALS, MDKLS, MNPLS, NELLS, NENLS, MVPLS, AMBLS and THNLS) and two isolates of leaf blight pathogens (MVPLB and NENLB) were obtained during the survey.

Leaf spot (VKALS)

Leaf spot (VKALS) was collected from Vellanikkara of Thrissur district. The variety cultivated was yellow passion fruit. The symptoms appeared as small, oval shaped, light coloured spots which later turned to brown colour. Prominent yellow halos were visible around the spots. The spots had an average size of one cm. Later, the centre of spots became brittle and fall off. Pin head like black fruiting bodies of the fungus, the acervuli were visible in the middle of older spots. The leaf spots were more numerous towards the leaf margin (Plate 3).

Leaf spot (MDKLS)

The leaf spot (MDKLS) was observed in Madakkathara of Thrissur district and the variety cultivated was yellow passion fruit. The disease was characterized by small minute brown spots of 0.3 - 0.5 cm on the leaves. The spots were seen throughout the leaf lamina. Later the spots enlarged and clear concentric zonations were visible on the spots. The spots coalesced and produced large blighted areas on the leaves. There was no yellow halo around the spots in the initial stages but developed afterwards (Plate 3).

Leaf spot (MNPLS)

The leaf spot, MNPLS was collected from Manjapra (Angamaly) of Ernakulam district and the variety cultivated at the location was yellow passion fruit. The isolate produced irregular shaped holes which were white in colour. The texture of the spots was papery and the infected areas fall off giving shot hole appearance at the later stages. Such spots were mainly seen on the mature leaves. Yellow halos were absent around the spots. Fructifications of the fruiting bodies were seen on spots in the advanced stages (Plate 3).

Leaf spot (MVPLS)

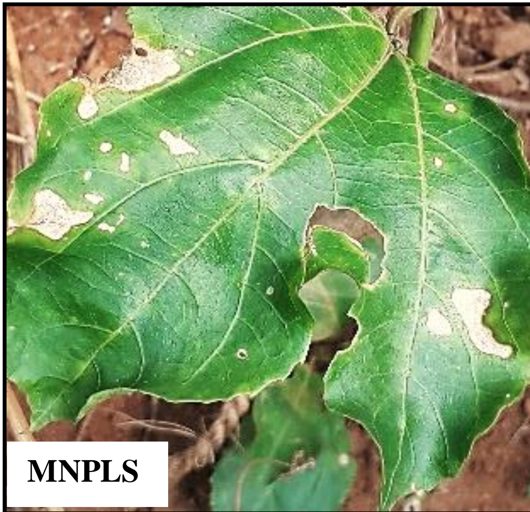
The leaf spot sample, MVPLS was collected from Muvattupuzha of Ernakulam district and the variety cultivated at the location was yellow passion fruit. Symptoms produced as large spots in the marginal areas of leaves. Average size of the spots was 2.0 cm length and 0.8 cm breadth. Drying of leaves from the marginal areas was observed which later produced necrotic patches with fruiting bodies. Severe chlorosis was observed on the infected leaves (Plate 3).

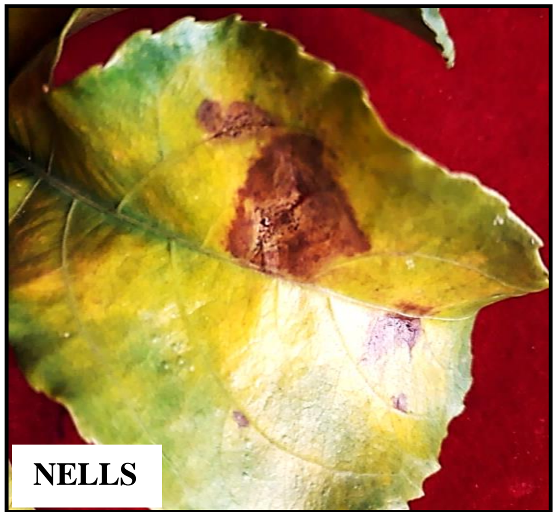
Leaf spot (NELLS)

Leaf spot (NELLS) was collected from Nelliyampathy of Palakkad district. The variety cultivated at the location was yellow passion fruit. The symptoms appeared as numerous small irregular spots at the initial stages. Later these spots coalesced to form necrotic patches and acervuli was visible in the advanced stages of disease at the center of spots. The diameter of spots ranged from 0.3 cm - 1.5 cm. Severe chlorosis was observed on the infected leaves. The symptoms were observed throughout the entire area of leaf lamina (Plate 3).

Leaf spot (NENLS)

The symptoms of the leaf spot isolate observed in yellow passion fruit of Nemmara location was initially appeared as white coloured spots with a brown margin. Later these spots turned to brown spots surrounded by yellow halo. The size of spots was 0.5 cm in length and 1.8 cm breadth. The spots were mostly seen on the interveinal





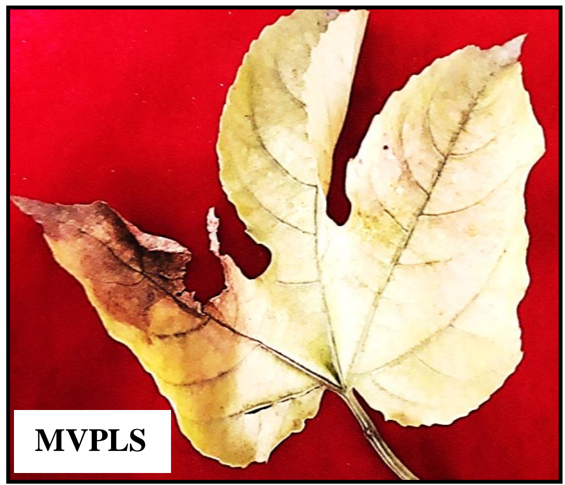


Plate 3: Symptomatology - leaf spots

areas of the leaves and fructifications of the fruiting bodies were visible at the centre of lesions (Plate 3).

Leaf spot (AMBLS)

The leaf spot sample (AMBLS) was collected from Ambalavayal and the variety cultivated was purple passion fruit. The symptoms appeared as creamy-white coloured, light spots of about 0.2 – 0.3 cm in diameter. Yellow halos were visible around the spots and light brown margins limited the spots. When the symptoms were produced on the marginal areas, the infected area dried and fall off (Plate 4).

Leaf spot (THNLS)

This leaf spot sample was collected from Thankamani, Idukki from yellow passion fruit variety. The symptoms produced were minute light coloured spots with brown margin which later enlarged and coalesced to produce extensive blighted areas on the leaf lamina. A narrow yellow halo surrounded the spots (Plate 4).

Leaf blight (MVPLB)

The leaf blight sample (MVPLB) was collected from Muvatttupuzha and the variety cultivated at the location was yellow passion fruit. The symptoms appeared as cream coloured small spots of about 0.1 - 0.3 cm diameter at the initial stage. These spots later enlarged and coalesced to form an irregular blighted appearance in the leaves. The spots were uniformly distributed over the entire leaf lamina (Plate 5).

Leaf blight (NENLB)

The leaf blight sample (NENLB) was collected from Nemmara of Palakkad district and the variety cultivated was yellow passion fruit. The symptom was observed as extensive blighted areas in the interveinal areas. The blighted areas were light coloured with papery texture and brittle nature. (Plate 5).

4.4.1.2 Bud blight (KLYBB)

Blighting of flower buds was recorded in yellow passion fruit variety from Kolazhy (Thrissur). The symptoms include blighting and drying of flower buds. Dark

brown coloured fruiting bodies were produced on the calyx of flower buds. Dropping of flower buds occurred on the advanced stage (Plate 6).

4.4.1.3 Fruit rots

Four types of fruit rot symptoms were recorded during the survey which were catalogued as MDKFR, NELFR, PAZFR and MVPFR.

Fruit rot (MDKFR)

The fruit rot (MDKFR) was collected from yellow passion fruit variety of Vellanikkara in Thrissur district. The symptoms appeared as light coloured sunken areas on the fruits with dark brown margins. The spots lacked water soaked appearance and finally produced dry rot of fruits. The size of spots ranged from 0.9 – 1.6 cm in diameter. Fructifications of fruiting bodies were not visible on the spots (Plate 7).

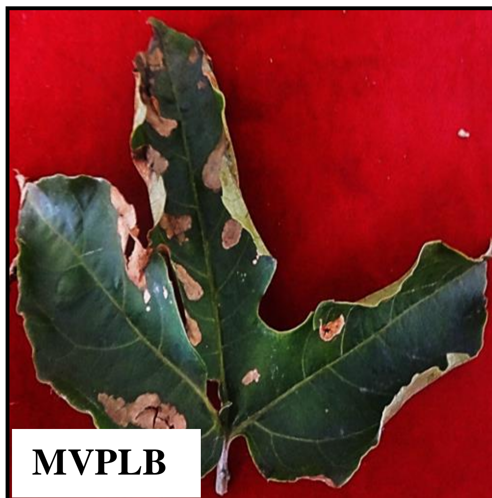
Fruit rot (NELFR)

The fruit rot (NELFR) was collected from Nelliyampathy of Palakkad district and the variety cultivated was yellow passion fruit. The symptoms appeared as dark brown coloured spots on the mature fruits. Later these spots turn into soft and sunken rotten areas. Lesions extended to large areas on the fruit and the skin separate from the fruit and become papery along with presence of acervuli at the centre of spots in the advanced stages. These spots affect the quality of pulp inside the fruits. (Plate 7)

Fruit rot (PAZFR)

This fruit rot disease was observed on yellow variety of passion fruit from the Pazhayannur farm of Thrissur district. It produced necrosis on the fruits. In these type of rotting, the initial symptom appeared as light pink coloured rotten areas with white cottony mycelial growth which later extended to produce extensive rotting of the fruits affecting the quality of fruit pulp. Sclerotial bodies were formed in the advanced stages of the diseases (Plate 7).

Plate 4: Symptomatology - leaf spots



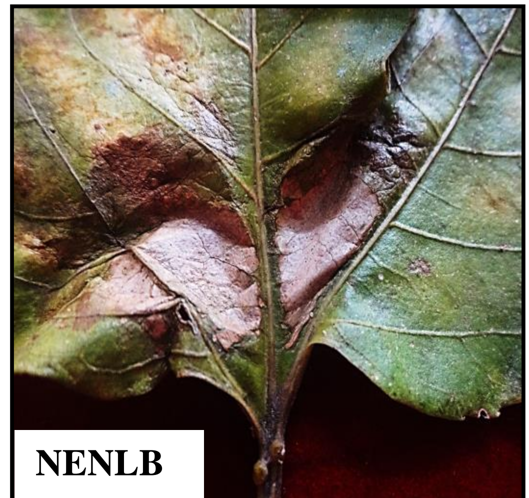


Plate 5: Symptomatology - leaf blights

Plate 6: Symptomatology - bud blight

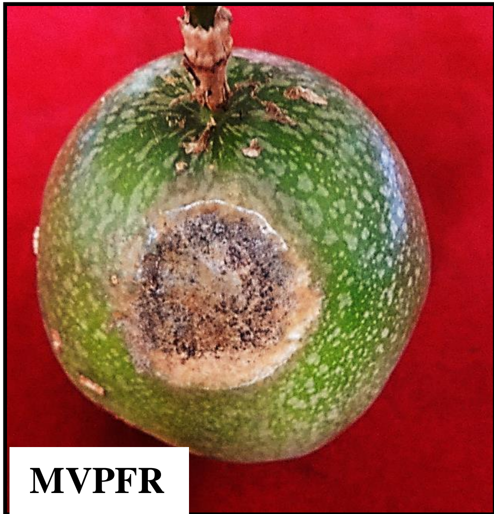


Plate 7: Symptomatology – fruit rots

Fruit rot (MVPFR)

The fruit rot sample (MVPFR) was observed in yellow passion fruit of Muvattupuzha, Ernakulam district. The symptoms were produced as light coloured spots which later enlarged to produce larger creamy white spots leading to the rotting of the fruits and black spore masses were seen on the spots in the advanced stages (Plate 7).

4.4.1.4 Wilting

Two isolates (MNPWt and KANWt) of wilt causing pathogen were collected during the survey from yellow passion fruit variety cultivated at the two locations, Manjapra and Kannara. Sudden wilting of the entire vines of passionfruit plant was observed as the initial symptom. The plants of all stages were infected but more incidence was observed in fruit bearing plants. The most prominent symptom was the rapid wilting of the entire vines while the leaves retained its green colour. Within few days of infection, the entire plant was wilted, dried spreading the infection to nearby vines. The other symptoms found associated with the diseases were discolouration of vascular bundles and extensive rotting of roots (Plate 8). Both the samples showed positive result for the ooze test.

4.4.1.5 Leaf malformation symptoms (VKALM)

Symptoms similar to the infection caused by virus were seen in a passion fruit orchard of Vellanikkara. The infection was extremely severe and majority of the vines were seriously affected with a variety of symptoms which were described below.

Islands of light green and dark green areas:

The patches of dark green and light green areas (Plate 9) with rugosity were observed on the leaves of passion fruit giving a mosaic like pattern. These were dark green plant tissues surrounded by light green plant tissues. The plants with such mosaic symptoms were stunted and less vigorous in growth.

Vein clearing:

Loss of chlorophyll in the areas of entire network of veins in the leaf blade was

the characteristic symptom of vein clearing (Plate 9) observed in passion fruit plants of the Vellanikkara, so that the veins became unnaturally clear and translucent. When the leaves were placed against sunlight, the symptoms were clearly visible.

Extreme reduction of leaf lamina:

The size of infected leaf lamina was reduced (Plate 9) as a result of infection and the leaves appeared misshaped.

Leathery appearance of leaf:

The natural texture of leaves was lost (Plate 10) and the flexibility was greatly reduced giving brittleness to the leaves. The infected leaves became light green coloured and stiff.

Puckering and cupping of leaf:

The leaves were distorted, puckered and crinkled (Plate 10). Puckering means the appearance of small pouch like structures on the leaves. Upward cupping of leaves was also seen. Puckering and cupping of leaves were mainly seen on newly emerging young leaves.

Fruit malformation:

Woody, malformed fruits were formed on the infected vines (Plate 10).

Chlorotic spots:

It was characterized by the uneven distribution of chlorophyll as yellow spots (Plate 9) over dark green patterns on the leaf. These spots were uniformly distributed among dark green tissues.

4.4.2 Symptoms under artificial inoculation

The symptoms under artificial inoculation were studied by Mycelial Bit Inoculation Method (MBIM) and the inoculated plant parts were observed for the initial appearance of symptom and progressive development of symptoms. The details of the symptoms developed on healthy plant parts by the inoculation of different isolates of the pathogen collected during the survey are given below.

Plate 8: Symptomatology (Wilting)

A. **Sudden wilting of entire vine (KANWt)**
B. **Sudden wilting (MNPWt)**
C. **Extensive rotting of roots**
D. **Internal discolouration of vascular bundles**
E. ***In situ* ooze test**
F. **Symptoms on artificial inoculation**
G. **Control plants**

Plate 9: Symptomatology – Leaf Malformation

A. Islands of dark green and light green areas
B. Chlorotic spots
C. Vein Clearing
D. Vein Clearing
E. Reduced leaf lamina

Plate 10: Symptomatology – Leaf Malformation

A. Puckering

B. Leathery appearance

C. Cupping

D. Malformed fruits

4.4.2.1 Foliage diseases

VKALS, MDKLS, MNPLS, MVPLS, NELLS, NENLS, AMBLS and THNLS produced typical leaf spot symptoms whereas MVPLB and NENLB produced blighting.

Leaf spot - VKALS

The pathogen associated with the leaf spot (VKALS) collected from Thrissur district (Vellanikkara) was inoculated on healthy leaves. The symptoms appeared on the leaves after two days of inoculation. The symptoms appeared as black lesions with water soaked appearance in the initial stages which later coalesced and developed yellow halos around the spot. The symptom progressed as an extensive spot of brown colour within one week of inoculation (Plate 11).

Leaf spot - MDKLS

The second leaf spot was collected from Madakkathara and was catalogued as MDKLS. The pathogen associated produced symptoms on inoculated leaves within four days. The spots showed typical black and brown water soaked lesions with alternate concentric zonations within one week of inoculation. No prominent yellow halo was seen in this case (Plate 11).

Leaf spot - MNPLS

MNPLS was the leaf spot collected from the Angamaly region of Ernakulam district. The fungus isolated from the sample produced symptoms within two days when inoculated on healthy leaves. Water soaked lesions with irregular shape were seen in the inoculated leaves and the infection spread at a very rapid rate covering the entire leaf lamina within five to six days of inoculation. There was no appearance of yellow halo around the infected leaves (Plate 11).

Leaf spot - MVPLS

The pathogen associated with the leaf spot (MVPLS) collected from Ernakulam district was inoculated on healthy leaves and produced symptoms within a period of two days. The symptoms initiated as small black water soaked lesions with a necrotic

appearance on the inoculated region. When the infection progressed, yellow halo was visible around the spots (Plate 11).

Leaf spot - NELLS

NELLS was the leaf spot collected from Nelliyampathy of Palakkad district and the pathogen associated with this leaf spot initiated the infection within three days of inoculation. The symptoms were appeared as light, brownish-black dark spots with an irregular growth pattern which later turned to a necrotic patch. A narrow yellow halo was seen around the spots (Plate 11).

Leaf spot - NENLS:

The leaf spot, NENLS was collected from a homestead in Nemmara of Palakkad district and the pathogen associated with the symptom showed the capability to infect the leaves within two to three days of inoculation. The initial water soaked lesion of black colour turned to a brown necrotic patch as the disease progressed. Prominent yellow halo was seen after one week of infection (Plate 11).

Leaf spot - AMBLS:

AMBLS was the leaf spot collected from Ambalavayal region of the Wayanad district. The pathogen associated with this symptom was capable of infecting the leaves by 2nd day after the inoculation. The inoculation period was very short. The symptoms appeared as minute black spots later enlarged to dark necrotic patches with water soaked appearance covering the entire leaf lamina (Plate 11).

Leaf spot - THNLS:

THNLS, the leaf spot isolate from Idukki district infected the leaves within two days of artificial inoculation. The symptoms appeared as black, water soaked spots with yellow halo round them. Later the spots coalesced to form necrotic patch covering the entire leaf lamina (Plate 11).

Leaf spot - MVPLB:

The foliar blight pathogen isolated from the leaf blight (MVPLB) collected from Muvattupuzha, Ernakulam district initiated the infection after four days on the

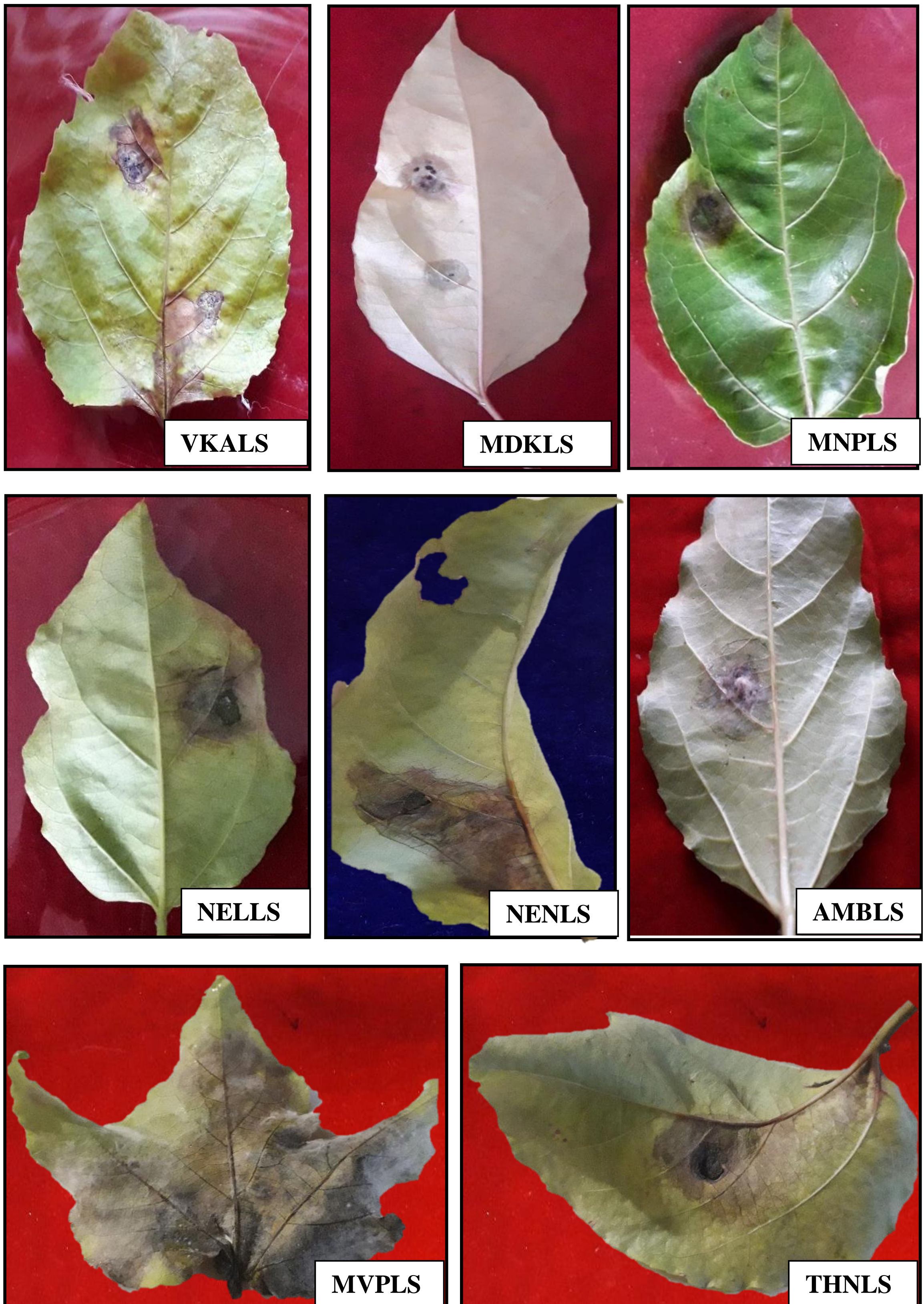


Plate 11: Symptomatology under artificial inoculation

inoculated leaves. The symptoms appeared as minute creamy white spots which later turned necrotic and coalesced to give a blighted appearance. There was no yellow halo around the infected areas. Later, as the infection progressed, the necrotic areas blown off giving shot hole appearance (Plate 12).

Leaf blight - NENLB:

The leaf blight pathogen isolated from the leaf blight sample (NENLB) collected from Nemmara region of Palakkad showed the capability to initiate infection within three days of inoculation. Minute cream coloured spots appeared in the initial stages and later enlarged to form patches of burned areas within one week of infection (Plate 12).

4.4.2.2 Fruit Rots

Four isolates causing fruit rots were collected during the survey and these were catalogued as MDKFR, NELFR, PAZFR and MVPFR.

Fruit rot - MDKFR:

The pathogen associated with the fruit rot (MDKFR) collected from an orchard in Madakkathara initiated the infection within four days of artificial inoculation. The symptom started as minute brown spots which later enlarged to spots of 2.33 cm length and 0.6 cm width on the 7th day and 4.6 cm length and 0.8 cm width on the 12th day of inoculation. The lesions appeared as greasy and light brown coloured with dark brown margins (Plate 12).

Fruit rot - NELFR:

The fruit rot pathogen isolated from the fruit rot sample (NELFR) collected from Nelliyampathy of Palakkad started the infection on the inoculated fruits within three days of inoculation. The symptoms appeared as small brown spots which enlarged to large water soaked brownish lesions finally leading to the entire rotting of the fruits (Plate 12).

Fruit rot - PAZFR:

The fungal pathogen isolated from the fruit rot (PAZFR) collected from state

seed farm of Thrissur (Pazhayannur) initiated the infection within two days of inoculation. The infection initiated as water soaked pink coloured spots which later enlarged and caused the rotting of entire fruit. White cottony mycelial mats appeared covering the fruits after the fourth day of inoculation. Sclerotial bodies appeared on the inoculated fruits after one week of inoculation (Plate 12).

Fruit rot - MVPFR:

The fruit rot pathogen isolated from the fruit sample (MVPFR) collected from Muvattupuzha region of Ernakulam district initiated the infection within three days of inoculation. The initial symptoms were brown spots which later enlarged and become sunken and dark brown. The lesion spread to the entire fruit and the skin become papery on progression of symptoms. Acervuli were formed on the fruit surface in the advanced stage (Plate 12).

4.4.2.3 Bud blight

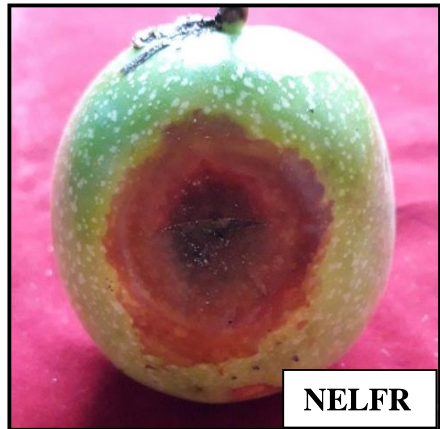
A single isolate of flower bud blight pathogen was isolated from the sample (KLYBB) collected from Kolazhy of Thrissur district. The pathogen started the infection on flower buds within 4 days of inoculation. Symptoms initiated as water soaked spots on the inoculated area which later resulted in the complete rotting of flower buds (Plate 12).

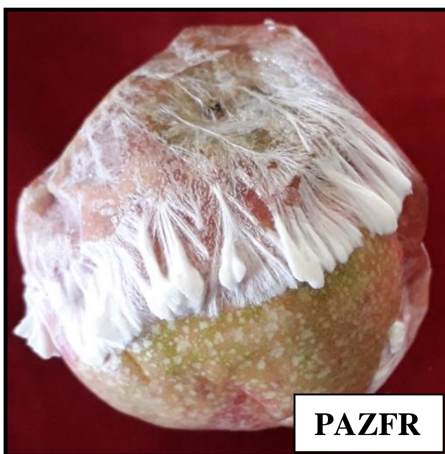
4.4.2.4 Wilting

Two isolates of bacterial pathogen causing wilt disease MNPWt and KANWt were collected during the survey. The pathogenicity of the disease was established using the protocol described in section 3.3.2. On artificial of the bacterial culture, the symptoms developed are given below.

Isolate MNPWt:

This bacterial wilt isolate was recorded in Manjapra (Angamaly) of Ernakulam district. The isolate produced typical wilting symptoms after four days of inoculation *i.e.* the leaves started to droop and green colour started to fade. Later, the tissues started to die and the inoculated plants withered completely within 10 days (Plate 8).





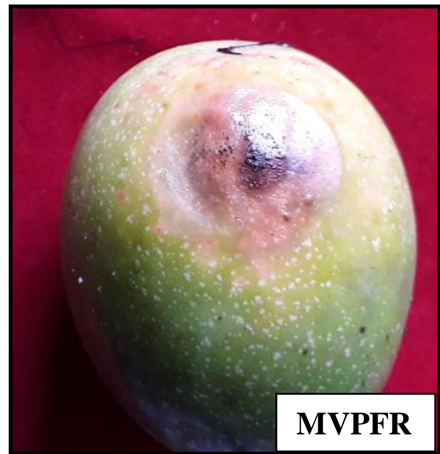


Plate 12: Symptomatology under artificial inoculation Cntd.

Isolate KANWt:

This isolate was recorded from Kannara of Thrissur district. On artificial inoculation, the wilting symptoms were produced after five to six days. The leaves started to droop and the green colour started to fade. Complete withering of plants occurred within two weeks.

4.5 CHARACTERISATION OF PATHOGENS

Isolation and pathogenicity studies revealed that the isolates causing leaf spots, leaf blights, bud blight and fruit rots as fungal pathogens and wilting was due to a bacterial pathogen. The leaf malformation was tentatively identified as a viral disease based on symptomatology.

4.5.1 Characterisation of fungal pathogens

The fungal pathogens isolated from infected samples of different locations were subjected to cultural and morphological characterisation for further identification of the fungus.

Based on the preliminary studies on isolation and pathogenicity, the isolates VKALS, MDKLS, NELLS, NENLS, MNPLS, MVPLS, AMBLS, THNLS, MVPLB, NENLB, MDKFR, NELFR, MVPFR and PAZFR were identified as fungal pathogens. These pathogens were further characterised for identification of each fungus.

4.5.1.1 Cultural and Morphological Characters

The cultural characters observed were colour, texture, growth rate (Table 4.5) pattern of growth, sporulations, colour on the back side of petri plates and presence of fruiting bodies. The various microscopic observations like colour of hyphae, branching pattern, sepatation, presence of conidia, conidial septation, types, shape and size of spores, length by breadth ratio of spores and presence of sexual structures were studied under morphological characters.

LEAF SPOT DISEASES

Eight leaf spot causing pathogen were isolated from the samples collected during the survey conducted in five districts of the state. *i.e.* VKALS, MDKLS,

ANGLS, MVPLS, NELLS, NENLS, AMBLS and THNLS.

Pathogen associated with Leaf spot 1 (VKALS):

This leaf spot was collected from Vellanikkara. On isolation, it produced white fluffy mycelial growth on PDA medium after 2 days of incubation. The culture was characterized by light grey aerial mycelium which gradually turned to dark grey coloured (Plate 13). Dark greyish black colour pigmentation was developed in the medium which was clearly visible at the reverse side of Petri plate. Later, orange coloured spore mass was developed on the mycelial growth. The growth of the pathogen covered the Petri plate (9 cm diameter) within seven days when incubated at room temperature and the growth rate was 1.29 cm per day (Table 4.5)

The morphological characters of the fungus were studied by slide culture technique followed by microscopic observations. This revealed that the hyphae were hyaline with septations and the conidia were single celled, hyaline, cylindrical, bullet shaped with round and obtuse ends and having a size of 8.64 µm(length) x 3.03 µm (breadth) (Plate 18).

Based on these cultural and morphological characters, the pathogen associated with the leaf spot disease 1 (VKALS) was identified at the genus level as *Colletotrichum* sp.

Pathogen associated with leaf spot 2 (MDKLS):

The fungus isolated from the second leaf spot sample from Thrissur district was inoculated on PDA medium in a Petri plate. It was characterized by the formation of sub surface mycelium with whitish grey colour and fungal colony was appeared within four days of incubation (Plate 13). The pathogen completed the growth in the Petri plate (9 cm diameter) by 11 days and the growth rate was 0.81 cm per day (Table 4.5). Alternate zones of black and cream colour were visible at the reverse side of the Petri plate.

The morphological characters were studied by microscopic observations and this revealed that the fungus produced brown coloured mycelium which were septate with abundant conidia production. The spindle shaped conidia were brown in colour

with transverse and longitudinal septa (muriform) having an average size of 18.28 µm length and 8.63 µm breadth and a prominent beak of 1.04 µm length (Plate 18).

With these cultural and morphological characters, the pathogen associated with the leaf spot disease 2 (MDKLS) was identified at the genus level as *Alternaria* sp.

Pathogen associated with Leaf spot 3 (MNPLS):

The pathogen associated with the leaf spot sample observed from Manjapra, Angamaly was isolated on PDA which produced white to smoky grey colonies on the medium within three days of incubation at room temperature. Fluffy white aerial growth of the mycelium was formed which later turned to greyish black followed by deep black colour (Plate 13). The culture completed full growth in the Petri plate (9 cm diameter) within five days and the growth rate of the pathogen was 1.8 cm per day (Table 4.5). The reverse side of the Petri plate appeared as deep black colour. Tough black coloured tarry structures were developed inside the Petri plates at the later stages.

Morphological characters of the pathogen were studied by the microscopic observations of the slide culture unit and this revealed that the hyphae were brown to black coloured and the isolate produced single celled conidia at the initial stage. The conidia were ellipsoidal or obovate with thick walls, brown coloured and developed single septa at the later stage which divided the conidia into two. The average size of the conidia was 18.32 µm length and 4.19 µm breadth (Plate 18).

Based on these cultural and morphological characters, the pathogen responsible for leaf spot 3 (MNPLS) was identified at the genus level as *Lasiodiplodia* sp.

Pathogen associated with Leaf spot 4 (MVPLS):

The pathogen associated with leaf spot sample collected from Muvattupuzha was isolated on PDA and produced fluffy dull white coloured mycelium which later turned to grey coloured (Plate 13). The pathogen completed the growth in Petr plate (9 cm diameter) within eight days and the growth rate was 1.13 cm per day (Table 4.5). The reverse side of the Petri plate showed dark grey coloured pigmentation.

The microscopic observation revealed that the fungus produced hyaline mycelium with septations. The conidia produced were bullet shaped with round and

obtuse ends. Oil globules were clearly seen at the centre of conidia. The average size of the conidia was found to be 7.38 µm length and 2.56 µm breadth (Plate 18).

The pathogen associated with MVPLS was identified at the genus level as *Colletotrichum* sp. based on these cultural and morphological characters.

Pathogen associated with Leaf spot 5 (NELLS):

The pathogen associated with leaf spot observed at Nelliyampathy of Palakkad district was isolated on PDA medium. It produced flat, submerged mycelial growth on the medium which was white in colour initially and later turned to grey colour (Plate 13). The fungus completed full growth within the Petri plate (9 cm diameter) in seven days and the growth rate was 1.29 cm per day (Table 4.5). The fungus produced cream coloured spore mass on the mycelia after two weeks of growth on the Petri plate and the reverse side of the petri plate appeared dark grey coloured.

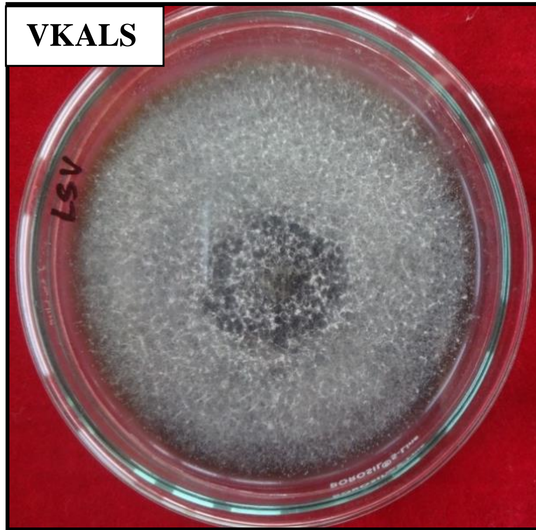
The microscopic observations revealed the morphological characters as the hyphae were hyaline initially later turned to greyish black and the conidia were hyaline and single celled having an oblong shape with round ends. The average size of the conidia was measured as 9.48 µm length and 3.57 µm breadth (Plate 18).

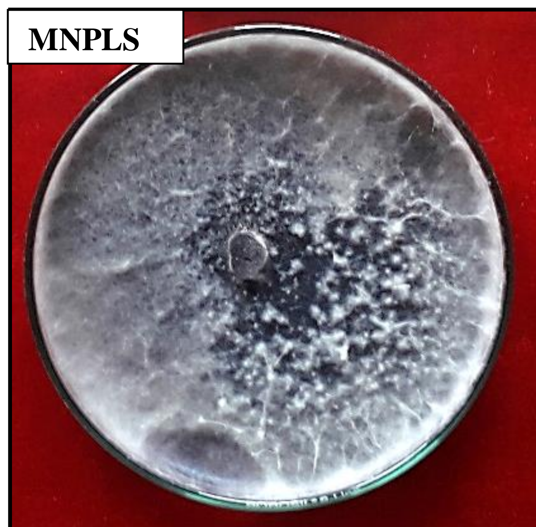
Based on these cultural and morphological characters, the pathogen associated with NELLS was identified at the genus level as *Colletotrichum* sp.

Pathogen associated with Leaf spot 6 (NENLS):

The leaf spot pathogen associated with NENLS collected from Nemmara of Palakkad district was isolated on PDA. The fungus produced submerged mycelium on PDA medium which was dark grey coloured and the fungus completed the growth in the Petri plate (9 cm diameter) within eight days (Plate 13). The growth rate was found out as 1.13 (Table 4.5). Cream coloured spore masses were produced later on the mycelial growth and the reverse side of the Petri plate appeared as dark grey coloured.

The morphological characters were studied by microscopic observations of the slide culture and which revealed that the hyphae were hyaline at the initial stages which later turned to grey coloured hyphae. Bullet shaped conidia were produced in the





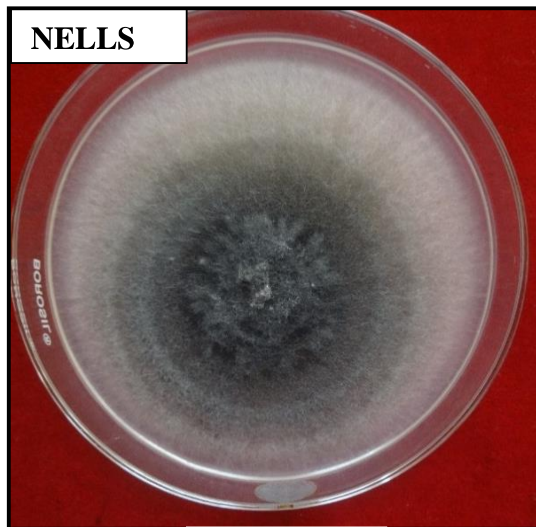
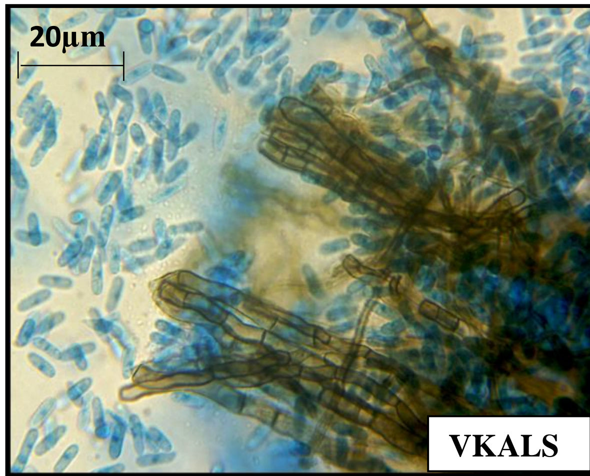
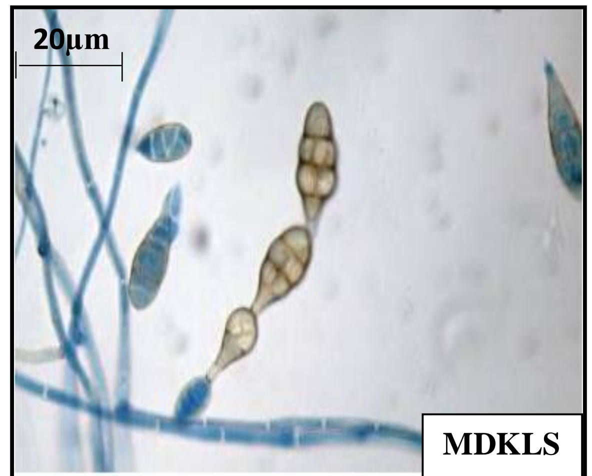
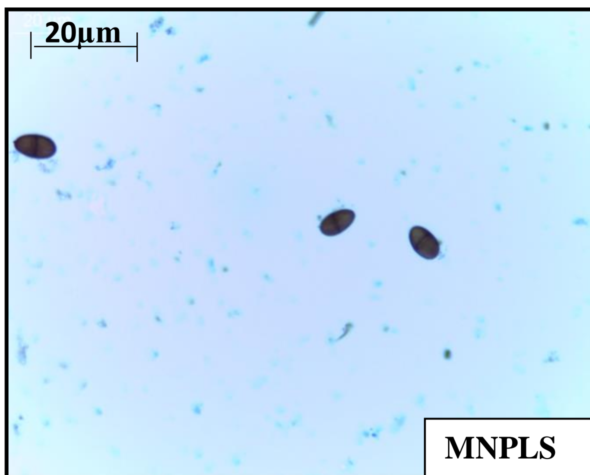
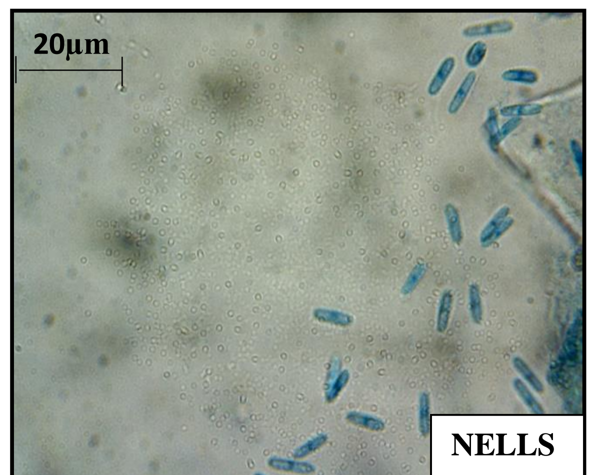


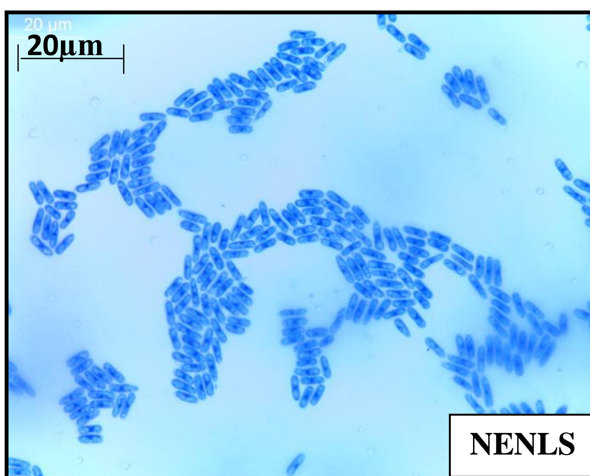
Plate 13: Cultural characters - leaf spot isolates











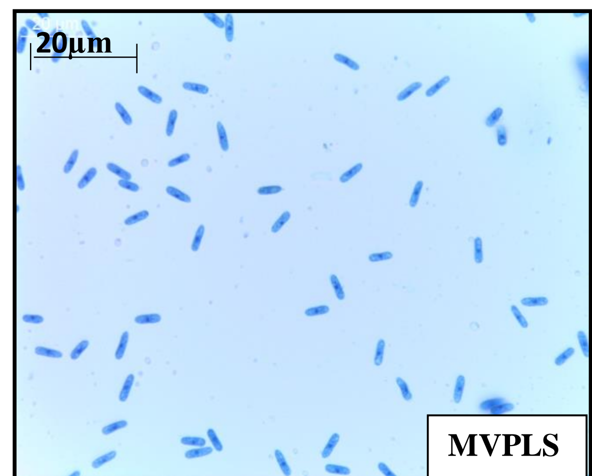


Plate 18: Morphological characters

medium with round ends and the average size of the conidia was measured to be 8.76 µm length x 2.67 µm breadth (Plate 18).

The pathogen associated with NENLS was identified at the genus level as *Colletotrichum* sp. based on these cultural and morphological characters.

Pathogen associated with Leaf spot 7 (AMBLS):

The pathogen associated with the leaf spot (AMBLS) collected from Wayanad was isolated on PDA medium. On isolation, it produced light grey coloured mycelium at the initial stage which later turned to dark grey colour (Plate 14). The fungus completed the growth within the Petri plate (9 cm diameter) in eight days and the growth rate was 1.13 cm per day (Table 4.5). Pink coloured spore mass was developed on the mycelial growth. Reverse side of the Petri plate was dark black in colour.

The microscopic observations revealed the morphological characters as the hyphae were hyaline with septations and the conidia were hyaline, bullet shaped and single celled with round ends. The average size of conidia was measured to be 6.9 µm length and 3.2 µm breadth (Plate 19).

Based on these cultural and morphological characters, the pathogen associated with AMBLS was identified at the genus level as *Colletotrichum* sp.

Pathogen associated with Leaf spot 8 (THNLS):

The pathogen responsible for the leaf spot (THNLS) collected from Idukki was isolated on PDA. On isolation, the fungus produced white to grey coloured colonies on the medium with a uniform growth pattern having regular margins (Plate 14). The reverse side of Petri plate appeared as dark grey coloured. The fungus attained complete growth of 9 cm in the Petri plate within eight days and the growth rate was 1.13 cm per day (Table 4.5).

The morphological characters were studied by slide culture technique and the microscopic observations of the same revealed that the hyphae were hyaline which gradually turned grey on ageing and become septate. Conidia were cylindrical in shape with round ends and the average size of conidia was measured to be 10.67 µm length x 3.06µm width (Plate 19).

Based on these cultural and morphological characters, the pathogen associated with THNLS was identified at the genus level as *Colletotrichum* sp.

LEAF BLIGHTS

Two isolates of fungal pathogen causing leaf blight were obtained by conducting the sampling survey in 5 districts.

Pathogen associated with Leaf Blight 1 (MVPLB):

The pathogen associated with leaf blight sample (MVPLB) collected from Muvattupuzha was isolated on PDA medium. It produced thin, pale white mycelium which later changed to brown colour (Plate 15). The pathogen showed a radial type of growth pattern on the medium. The colony diameter reached complete growth in the Petri plate (9cm diameter) within three days of inoculation which showed that the pathogen was fast grower and the growth rate was 3 cm per day (Table 4.5).

The microscopic observations revealed that the hyphae were septate and appeared hyaline at the initial stages which later turned to light brown colour after seven days and the branches arised at right angles below the septa (Plate 19).

These cultural and morphological observations confirmed that the pathogen associated with MVPLB as *Rhizoctonia* sp. at the genus level.

Pathogen associated with Leaf Blight 2 (NENLB):

Pathogen associated with leaf blight sample (NENLB) collected from Nemmara produced pure white colonies expanding in a zonate manner with radial growth and the mycelium turned light brown on ageing when isolated on PDA medium (Plate 15). The pathogen completes the growth within Petri plate in four days after incubation and the growth rate was 2.25 (Table 4.5). Dark brown sclerotia like bodies with irregular shape developed on the culture when it matures.

The morphological characters when studied by slide culture technique revealed that the hyphae appeared hyaline with septations and the branches arised at right angle below the septa with distinct constrictions (Plate 19).

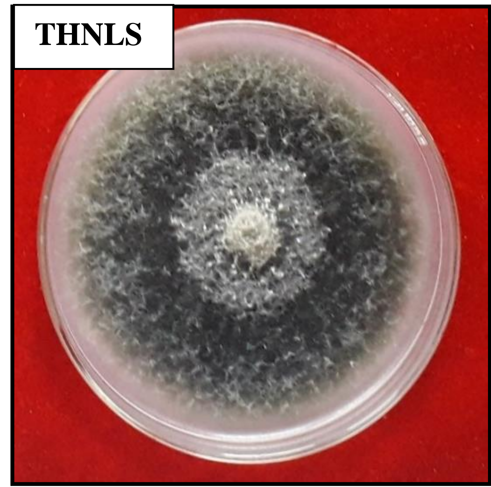
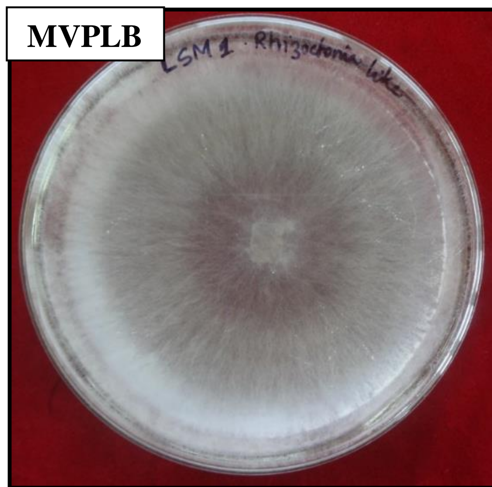


Plate 14: Cultural characters - leaf spot isolates



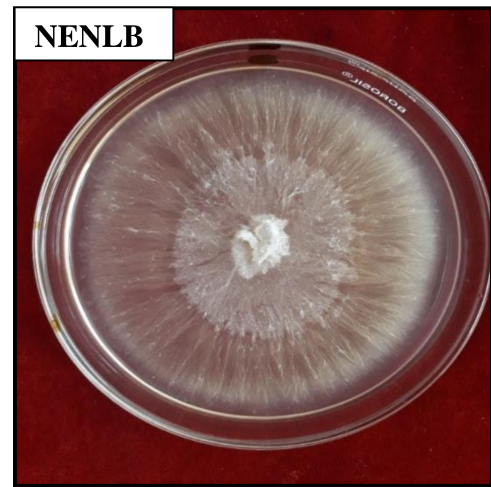
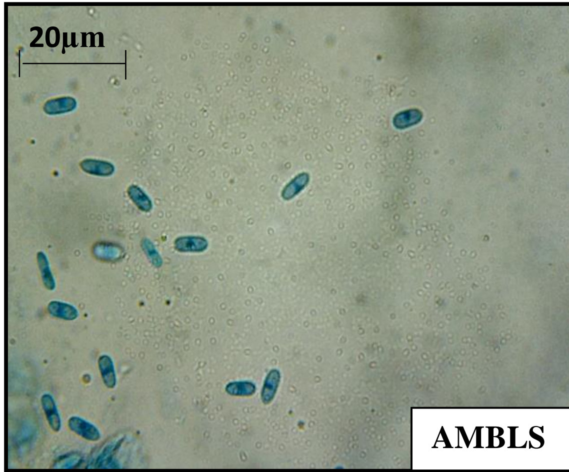
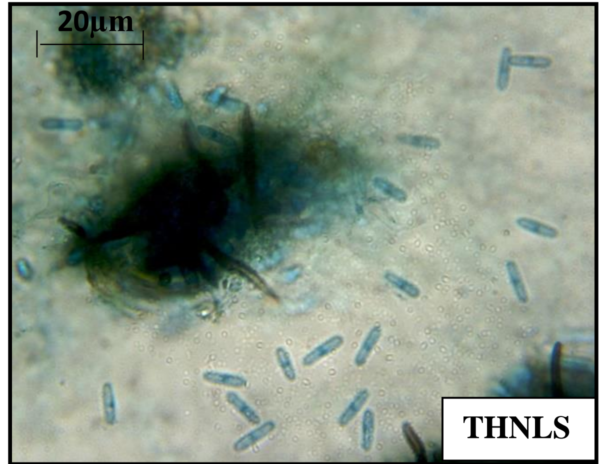
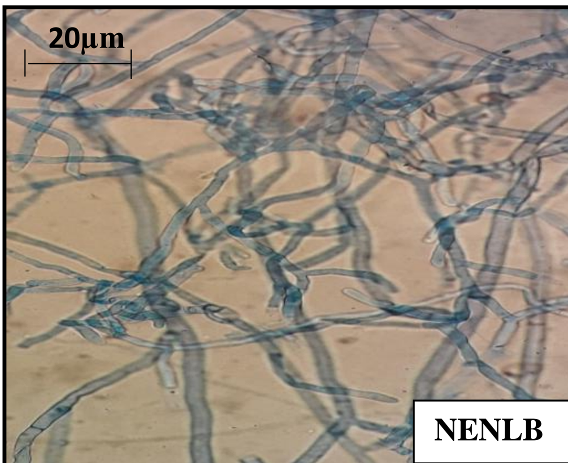


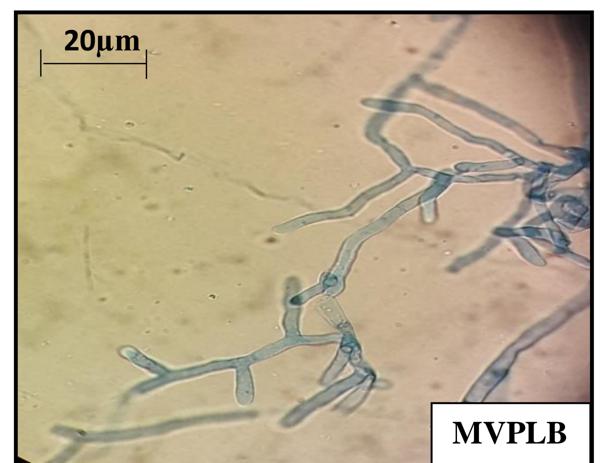
Plate 15: Cultural characters - leaf blight isolates

Plate 16: Cultural characters – bud blight isolate









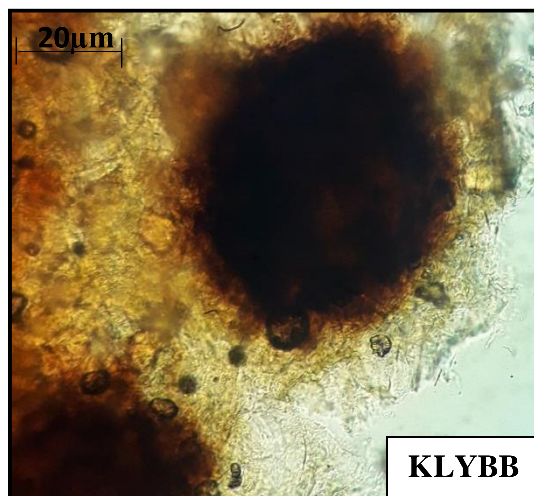


Plate 19: Morphological characters

Based on these cultural and morphological characters, the pathogen associated with NENLB was identified at the genus level as *Rhizoctonia* sp.

FLORAL DISEASES

Pathogen associated with bud blight (KLYBB):

The isolation of pathogen associated with bud blight (KLYBB) on PDA yielded a fungus with white coloured submerged mycelium. The pathogen completed the growth within six days on Petriplate (9 cm diameter). Dark brown to black coloured pycnidia were produced on the culture after 15 days of incubation (Plate 16).

Morphological characters studied by slide culture technique and microscopic observations revealed that the fungus produced hyaline and septate mycelium. Asexual fruiting bodies (pycnidium) of the fungus were visible under the microscope (40 X) with a dimension of 80.45 µm X 74. 65 µm (Plate 19).

These cultural and morphological characters revealed that the pathogen associated with KLYB as *Phomopsis* sp. at the generic level.

FRUIT ROT DISEASES

Four isolates of fruit rot pathogen collected from different locations of the survey *i.e.* MDKFR, NELFR, PAZFR and MVPFR.

Pathogen associated with Fruit rot 1 (MDKFR):

This fruit rot causing pathogen was isolated from the sample collected from Madakkathara and the fungus was characterized by the sub surface growth of mycelium on PDA medium with a dull whitish - grey colour (Plate 17). The pathogen completed the growth in the Petri plate (9 cm) by 12 days and the growth rate was 0.75 cm per day (Table 4.5). Alternate zones were clearly visible at the reverse side of the Petri plate with cream and blackish colour.

The microscopic observations revealed that the fungus produced brown coloured mycelium which were septate. Abundant conidia production occurred on aging of culture. The spindle shaped conidia were brown in colour with transverse and

longitudinal septa (muriform) with an average size of 18.87 µm length and 9.34 µm width and a prominent beak of 0.97 µm length (Plate 20).

Based on these cultural and morphological characteristics, the pathogen associated with MDKFR was identified at the genus level as *Alternaria* sp.

Pathogen associated with fruit rot 2 (NELFR):

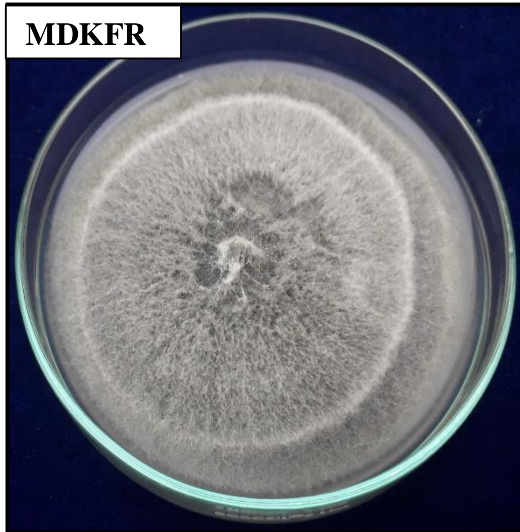
The pathogen associated with fruit rot sample collected from Nelliyampathy of Palakkad district was isolated on PDA medium and it produced appressed and submerged growth of mycelium on the medium which was white coloured initially and later turned grey in colour (Plate 17). Full growth of fungus was produced within the Petri plate (9 cm diameter) within a week and the growth rate was 1.29 cm per day (Table 4.5). The fungus produced cream coloured spore mass after two weeks inside the Petri plate. The reverse side of the petri plate appeared dark grey coloured.

The microscopic observations of the slide culture unit carried out to study the morphological characters revealed that the hyphae were hyaline at the initial stage which later turned to greyish black. The conidia were hyaline and single celled having an oblong shape with round ends and oil globules were clearly visible at the centre. The average size of the conidia was measured to be 10.32 µm length x 3.01 µm breadth (Plate 20).

Based on these cultural and morphological characters, the pathogen associated with NELFR was confirmed at the genus level as *Colletotrichum* sp.

Pathogen associated with fruit rot 3 (PAZFR):

The pathogen associated with fruit rot sample (PAZFR) collected from Pazhayannur region of Thrissur district was isolated on PDA medium and the fungus produced pure white aerial mycelium on the medium and the pattern of growth was in a radial manner (Plate 17). The pathogen completed full growth in the Petri plate (9 cm diameter) within four days of incubation and the growth rate was 2.25 cm per day (Table 4.5). Sclerotial bodies were started to appear in the Petri plate within ten days. They appear smooth, perfectively round with smooth shiny surface and the colour was



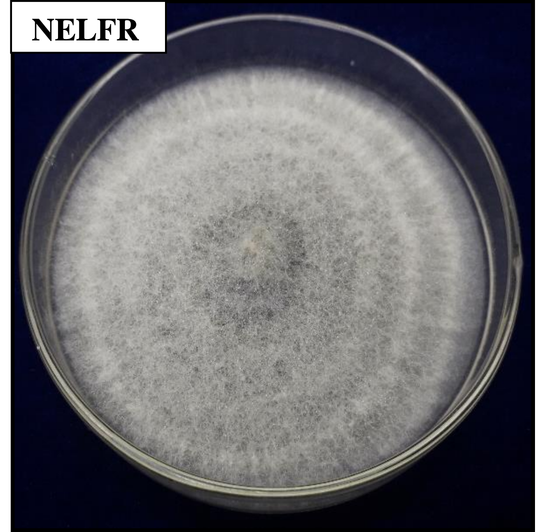
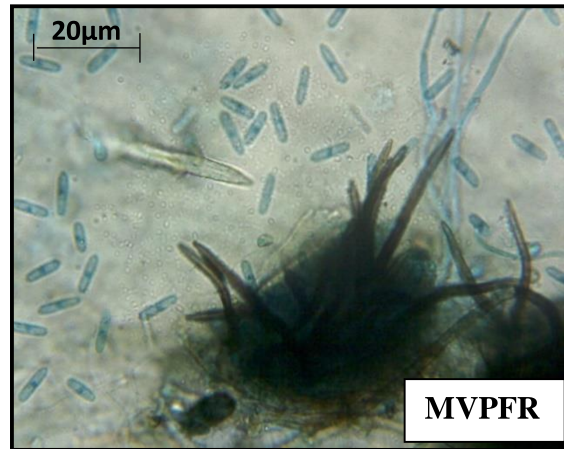


Plate 17: Cultural characters – fruit rot isolates



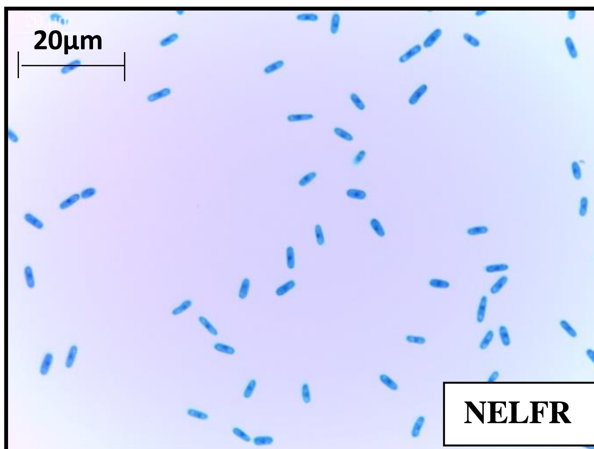


Plate 20: Morphological characters

yellowish initially turning to dark brown (Plate 20). On the reverse side of petri plate appeared dull white to yellow colour.

The morphological characters studied by slide culture technique and microscopic observations revealed that the hyphae were hyaline and septate. On the basis of these cultural and morphological characters, the pathogen associated with PAZFR was confirmed as *Sclerotium* sp. at the genus level.

Pathogen associated with fruit rot 4 (MVPFR):

The pathogen associated with fruit rot (MVPFR) collected from Muvattupuzha was isolated on PDA medium. On isolation, the fungus produced dull white coloured fluffy mycelium which later turned to grey coloured (Plate 17). The pathogen completed full growth in the Petri plate within eight days and the growth rate was 1.13 cm per day (Table 4.5). the reverse side of the petri plate appeared dark grey colour.

The morphological characters studied by slide culture technique and microscopic observations revealed that the fungus produced hyaline and septate mycelium. The conidia produced were bullet shaped with round and obtuse ends. Oil globules was clearly visible at the centre of conidia. The average size of the conidia was found to be 8.08 µm length and 2.49 µm breadth (Plate 20).

Based on these cultural and morphological characters, the pathogen associated with MVPFR was identified at the genus level as *Colletotrichum* sp.

4.5.2 Characterisation of Bacterial pathogens

Bacterial isolates collected during the survey conducted in various passion fruit growing areas of five districts of the state were characterised and identified on the basis of cultural, morphological and biochemical characteristics. The cultural characteristics like colour and texture of bacterial colonies on artificial medium, pigmentation and mucoidal nature of the bacterial colonies were observed for both the isolates of the bacterium. The morphological characters like size and shape of the bacterial cells were studied by observing the bacterial cells under ordinary light microscope (1000 X) and by Electron microscopy (10800 X and 16500X).

Isolates	Colony diameter (cm)												Days for full growth	Growth rate
	Days of inoculation													
	1	2	3	4	5	6	7	8	9	10	11	12		
VKALS	1.5	2.9	4.0	6.3	7.6	8.6	9.0						7	1.29
MDKLS	-	0.9	1.4	2.6	3.4	4.7	5.8	6.6	7.7	8.4	9.0		11	0.81
NELLS	1.3	3.1	4.2	5.9	7.4	8.3	9.0						7	1.29
NENLS	0.8	2.4	3.2	4.7	5.9	7.1	8.4	9.0					8	1.13
MNPLS	0.4	2.6	5.2	8.4	9.0								4	1.8
MVPLS	1.2	2.7	3.4	5.2	6.7	7.9	8.5	9.0					8	1.13
THNLS	1.4	2.8	3.7	5.6	6.9	7.6	8.6	9.0					8	1.13
AMBLS	1.3	2.6	3.8	5.4	6.7	7.7	8.4	9.0					8	1.13
NENLB	2.3	4.9	7.8	9.0									4	2.25
MVPLB	2.1	5.7	9.0										3	3.00
MDKFR	-	0.4	1.1	1.9	2.6	3.4	4.9	5.7	6.9	7.6	8.5	9.0	12	0.75
NELFR	1.1	2.7	3.9	5.3	6.5	8.3	9.0						7	1.29
MVPFR	1.5	2.9	4.2	5.4	6.6	7.9	8.6	9.0					8	1.13
PAZFR	2.0	4.8	8.1	9.0									4	2.25
KLYBB	1.2	3.6	5.8	7.0	8.6	9.0							5	1.80

Table 4.5: Growth rate of different fungal isolates on PDA

Table 4.6: Cultural and morphological characters of Fungal isolates

Isolate	Cultural characters	Reverse side of Petri plate	Morphological characters				Associated pathogen
			Hyphae	Shape of conidia	Colour of conidia	Dimension of conidia	
VKALS	Fluffy white mycelium gradually turned dark grey colour	Greyish black colour	Hyaline, septate	Bullet shaped	Hyaline	8.64 µm x 3.03 µm	*Colletotrichum* sp.
MDKLS	Sub surface mycelium of whitish grey colour	Alternate zones of black and buff colour	Brown, septate	Muriform	Brown	18.28 µm x 8.63 µm	*Alternaria* sp.
NELLS	Flat, submerged, white mycelium later turned to grey colour	Dark grey colour	Hyaline, septate	Bullet shaped	Hyaline	9.48 µm x 3.57 µm	*Colletotrichum* sp.
NENLS	Submerged, dark grey colour mycelium	Dark grey	Hyaline	Bullet shaped	Hyaline	8.76 µm x 2.67 µm	*Colletotrichum* sp.
MNPLS	Fluffy, white to smoky grey colonies later turned to greyish black	Deep black	Brown to black, septate	Ellipsoid	Brown	18.32 µm x 4.19 µm	*Lasiodiplodia* sp.
MVPLS	Fluffy, dull white Mycelium later turned to grey colour	Dark grey	Hyaline, septate	Bullet shaped	Hyaline	7.38 µm x 2.56 µm	*Colletotrichum* sp.
THNLS	Fluffy, white to grey colour	Dark grey	Hyaline, septate	Cylindrical	Hyaline	10.67 µm x 3.06µm	*Colletotrichum* sp.

Table 4.6: Cultural and morphological characters of Fungal isolates (Cntd...)

Isolate	Cultural characters	Reverse side of Petri plate	Morphological characters				Associated pathogen
			Hyphae	Shape of conidia	Colour of conidia	Dimension of conidia	
AMBLS	Fluffy, light grey mycelium later turned to dark grey colour	Black colour	Hyaline, septate	Bullet shaped	Hyaline	6.9 µm x 3.2 µm	*Colletotrichum* sp.
NENLB	Pure white colonies in a zonate manner later turned light brown	Buff colour	Hyaline, septate	Dark brown sclerotia developed with irregular shape hyphal branches arised at right angle			*Rhizoctonia* sp.
MVPLB	Thin, white mycelium in a radial type growth later turned to brown	Dark grey colour	Hyaline, septate	Hyphal branches arised at right angles below the septa			*Rhizoctonia* sp.
MDKFR	sub surface mycelial growth of dull whitish - grey colour	Alternate zones of black and buff colour	Brown, septate	Muriform	Brown	18.87 µm x 9.34 µm	*Alternaria* sp.
NELFR	Appressed, white mycelial growth later turned grey	Dark grey	Hyaline, septate	Oblong shape	Hyaline	10.32 µm x 3.01 µm	*Colletotrichum* sp.
	Fluffy, dull white mycelium later turned to grey colour	Dark grey	Hyaline, septate	Bullet shaped	Hyaline	8.08 µm x 2.49 µm	*Colletotrichum* sp.
PAZFR	Pure white aerial mycelium growing in a radial manner	White to yellow colour or buff colour	Hyaline, septate	Sclerotial bodies were smooth, perfectively round with shiny surface and the colour was yellowish initially turning to dark brown			*Sclerotium* sp.
KLYBB	White submerged mycelium later developed dark coloured pycnidium	Buff colour	Hyaline, septate	Dark brown to black coloured pycnidia were developed on the culture in a scattered manner after 15 days of incubation.			*Phomopsis* sp.

4.5.2.1 Cultural Characterisation

Two isolates of bacterial wilt pathogen *viz.* MNPWt and KANWt were collected from Manjapra of Ernakulam district and Kannara of Thrissur district respectively during the survey. Both the isolates produced colonies after 48 h of incubation. The isolation of these isolates on nutrient agar showed smooth, round, off white colonies with mucoid appearance and fluidal texture (Plate 21).

When grown on Tetrazolium Chloride medium specific to *Ralstonia solanacearum* showed typical fluidal white coloured colonies of bacteria with reddish pink centre (Plate 21). The colony characters were similar to that of *Ralstonia solanacearum*.

4.5.2.2 Morphological Characterisation

The morphology of bacterial cells when studied under ordinary light microscope in 1000 X magnification revealed that the isolates were rod shaped bacteria.

The bacterial cell morphology was observed using scanning electron microscope (Tescan Vega-3 LMU) at Central Instrumentation Laboratory, College of Veterinary and Animal Science, Mannuthy. Electron microscopic image revealed that the pathogenic isolates causing wilt was a rod shaped bacterium and the size of the bacterium were in the range 1.14 to 1.9 µm (Plate 21). An optimum working distance of 4.99 mm and voltage of 10kV was standardised by trial and error method for taking high resolution image for the bacteria.

These morphological characters were similar to that of *Ralstonia solanacearum*.

4.5.2.3 Biochemical Characterisation

The biochemical tests like gram reaction, starch hydrolysis, gelatin hydrolysis, casein hydrolysis, urease test, indole production test, citrate test and oxidase test were performed for the isolates for further confirmation of the identity through its reaction to various biochemical tests.

Gram staining:

When Gram staining was performed, the cells appeared as pink by taking up the counter stain i.e. safranin. This was due to the formation of perforations in the

cell wall since the lipid content of bacterial cell wall was readily dissolved by alcohol, which is a typical character of gram negative bacteria. This Gram's test revealed that the pathogen associated with wilt disease in passion fruit was gram negative bacterium which confirms the identity as *Ralstonia solanacearum*.

Amylose production test:

The isolates of wilt pathogen were capable of producing the enzyme, Amylose. Therefore, a clearing zone (Plate 22) was formed in the medium indicating the utilization of starch when iodine was added because starch in the presence of iodine produces a dark blue coloration of the medium and a yellow zone around the bacterial colony indicated the amylose activity. Das and Chattopadhyay (1955) observed the amylose production activity of *R. solanacearum* (Table 4.7).

Gelatin Hydrolysis test:

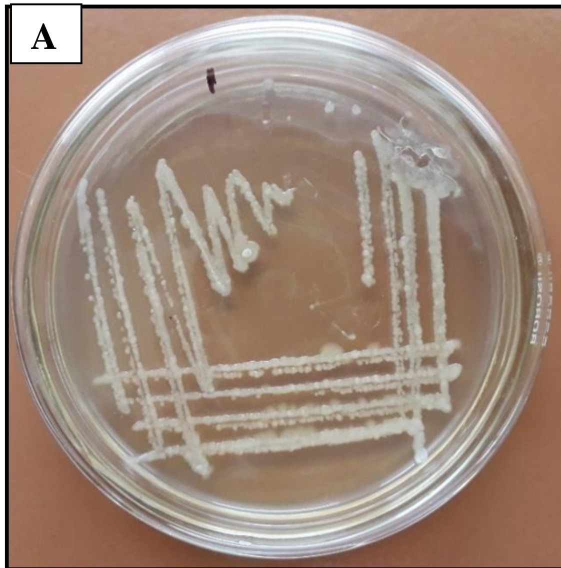
The two bacterial isolates collected from the wilt samples showed negative reaction (Plate 22) towards the gelatin hydrolysis *i.e.* they are not capable of producing the enzyme gelatinase (Table 4.7). Therefore, the gelatin medium inoculated with bacterial isolates solidified after keeping in 4°C for few minutes. *R. soanacearum*, also showed negative reaction towards gelatin hydrolysis (Sharma and Singh (2019).

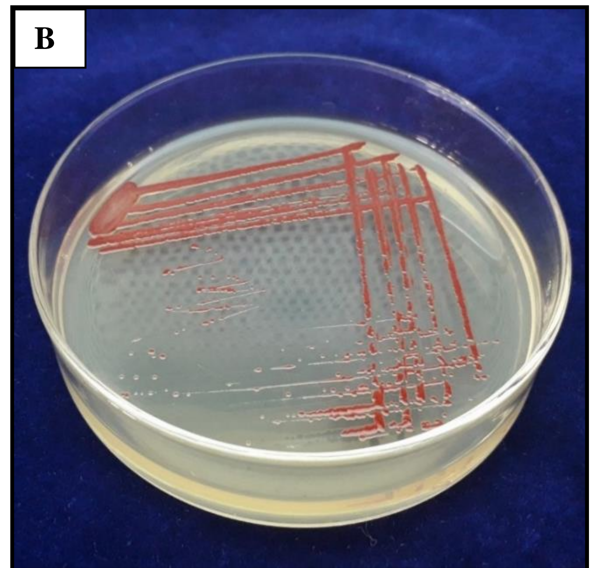
Casein Hydrolysis test:

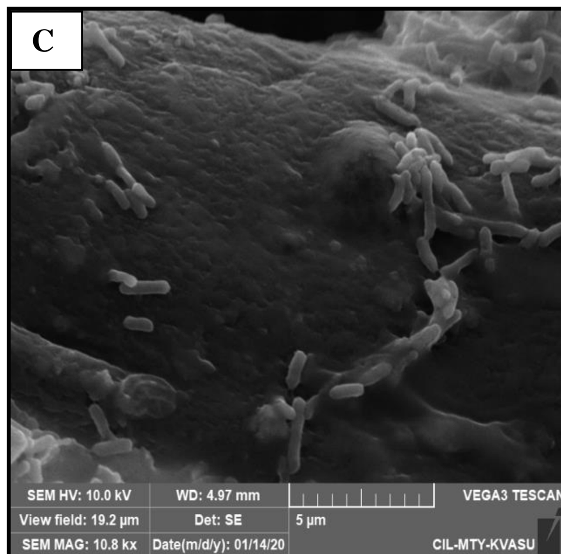
The bacterial isolates were capable of degrading casein in the medium due to the ability to produce caseinase enzyme. Therefore, a clear zone was formed around bacterial growth (Plate 22) after incubating for about 48 h at room temperature in an inverted position (Table 4.7). Singh *et al.* (2015) revealed that *R. solanacearum* was capable of producing caseinase enzyme.

Urease test:

The two bacterial isolates (MNPWt and KANWt) were capable of producing urease enzyme. Because, the urea agar slants inoculated with these isolates produced a pink colour (Plate 22) after incubating for two days due to the increase in pH by the liberation of ammonia due to urease production (Table 4.7). The urease activity of *R. solanacearum* was earlier described by Shekhawat *et al.* (2000).







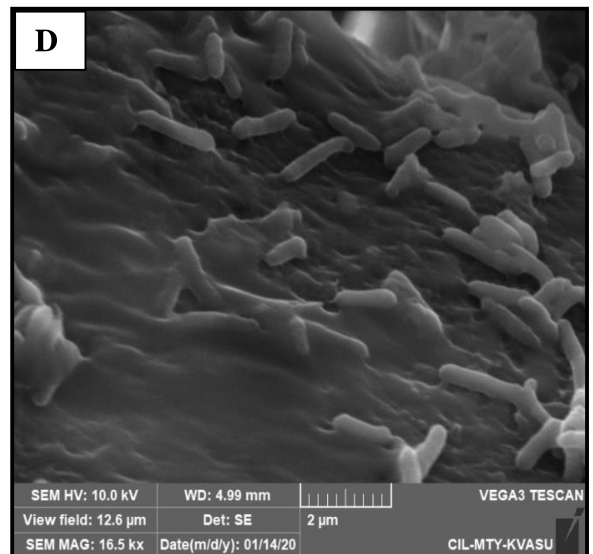


Plate 21: Cultural and morphological characterisation of bacteria associated with wilting

A. **Cultural characters on nutrient agar**
B. **Cultural characters on TZC**
C. **Scanning Electron Micrograph at 10.8 kX**
D. **Scanning Electron Micrograph at 16.5 kX**

Indole production:

The bacterial isolates showed negative reaction towards the indole production. Deep cherry red layer was not formed (Plate 22) due to the absence of indole production when Kovac's reagent was added on to tryptone broth (1%) inoculated with the bacterial cultures (Table 4.7). Seleim *et al.* (2014) observed that *R. solanacearum* was not capable of producing indole and showed a negative reaction on addition of Kovac's reagent.

Citrate Test:

The bacterial isolates collected from Manjapra, Angamaly(MNPWt) and Kannara (KANWt) were citrate positive (Plate 22) *i.e.* these bacteria were capable of utilizing the citrate in the medium and there by produced carbon dioxide which increased the pH and blue colour was developed in the inoculated slants (Table 4.7). Citrate utilization by *R. solanacearum* was observed by Sharma and Singh (2019).

Oxidase test:

The two isolates of bacterial wilt were capable of producing cytochrome C oxidase (Oxidase positive). Therefore, the reagent tetramethyl-P-phenylene diamine present on the oxidase disc was converted into indophenols which is the purple coloured end product (Plate 22 and Table 4.7). Hossain *et al.* (2007) revealed that *R. solanacearum* showed a positive reaction towards oxidase test.

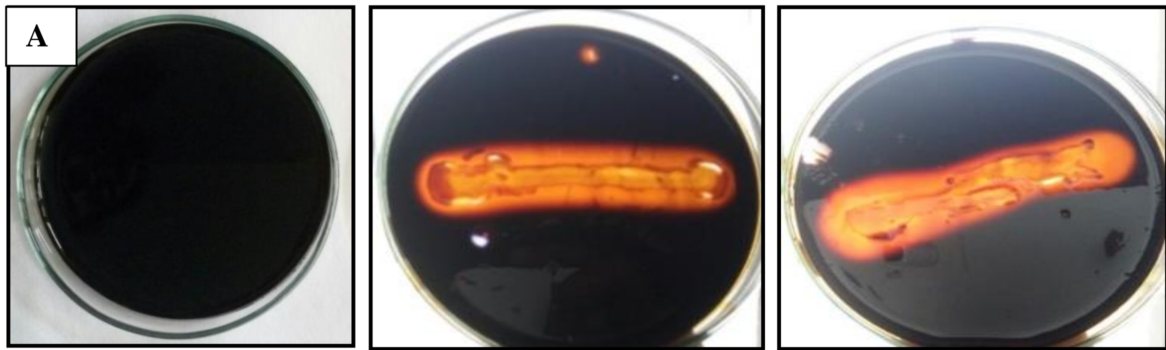
Based on the reaction of these isolates towards different biochemical tests, the isolates were confirmed to be *R. solanacearum.*

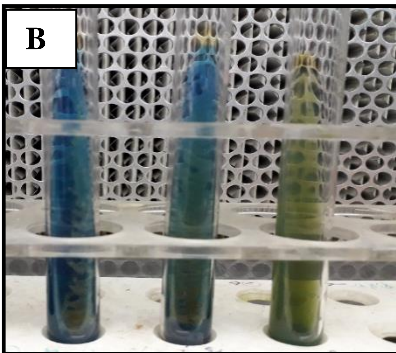
4.5.2.4 Identification of Races

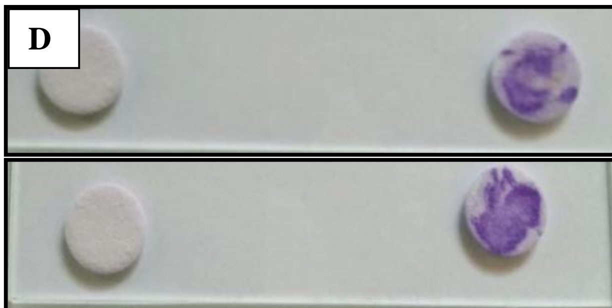
The two isolates of *R. solanacearum* (MNPWt and KANWt) collected from passion fruit wilt samples were inoculated into differential host plants *viz.,* tomato, brinjal, chilli, banana, potato, ginger and mulberry. The isolates; MNPWt and KANWt produced wilting symptoms on Tomato, brinjal and chilli within one week. However, these isolates failed to produce wilting symptoms on other differential hosts like banana, ginger, mulberry and potato (Plate 23). Therefore, based on the infectivity on solanaceous crops these isolates were identified as Race 1.

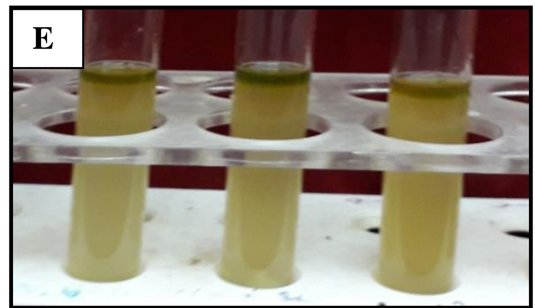
Table 4.7: **Reaction of bacterial isolates towards biochemical tests**

Sl. no.	Biochemical test	Reaction		Reaction of *R. solanacearum*	Reference
		MNPWt	KANWt		
1.	Amylose production test	+	+	+	Das and Chattopadhyay (1955)
2.	Hydrolysis of Gelatin	-	-	-	Sharma and Singh (2019)
3.	Casein hydrolysis	+	+	+	Singh *et al.* (2015)
4.	Urease test	+	+	+	Shekhawat *et al.* (2000)
5.	Indole production	-	-	-	Seleim *et al.* (2014)
6.	Citrate test	+	+	+	Sharma and Singh (2019)
7.	Oxidase test	+	+	+	Hossain *et al.* (2007)









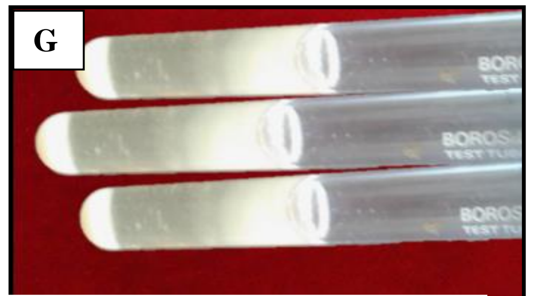


Plate 22: Biochemical characterisation of bacteria associated with wilting

A. Starch hydrolysis test **B. Citrate test**
C. Urease test **D. Oxidase test**
E. Indole production test **F. Casein hydrolysis test**
G. Gelatin hydrolysis test

BRINJAL

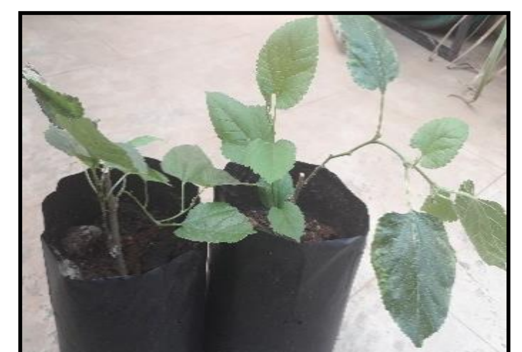
CHILLI

TOMATO

GINGER

POTATO

BANANA



MULBERRY

Plate 23: Race identification of bacteria associated with wilting

Table 4.8: Identification of Races

Differential hosts	**Isolates**	
	MNPWt	**KANWt**
Tomato	Wilted	Wilted
Brinjal	Wilted	Wilted
Chilli	Wilted	Wilted
Banana	No symptom	No symptom
Potato	No symptom	No symptom
Ginger	No symptom	No symptom
Mulberry	No symptom	No symptom
Race	**1**	**1**

4.5.3 Characterisation of Virus

The virus associated with leaf malformation (VKALM) symptom collected from Vellanikkara was characterised by sap transmission, graft transmission and the morphology of virus particles were studied by Transmission Electron microscopy.

4.5.3.1 Biological Characterisation

To check whether the virus associated with leaf malformation is mechanically transmissible or graft transmissible, biological characterisation was done.

Sap transmission

The experiment was conducted on newly emerged cotyledonous leaves of cowpea seedlings by following the protocol in section 3.5.3.1.1 under insect proof net house.

After 4 days, necrotic local lesions (Plate 24) were developed on the inoculated leaves confirming the virus is mechanically transmissible. The results revealed that the virus associated with leaf malformation of passion fruit is sap transmissible and 100 per cent transmission rate was recorded.

Table 4.9: Mechanical transmission of virus

Test Plant	No. of plants		Types of symptoms	Per cent Transmission	Incubation period of virus
	Inoculated	Infected			
Cow pea	10	10	Necrotic lesion	100	4 days

Graft transmission

Cleft grafting was performed using scions showing typical symptoms taken from infected plants on 4 months old root stock to check whether the virus can be transmitted by grafting. A total of 10 healthy root stocks were grafted with infected scions. Five grafts were successful and all the, successful graft unions resulted in cent per cent transmission of virus (Plate 24). The symptomatic plants on back inoculation

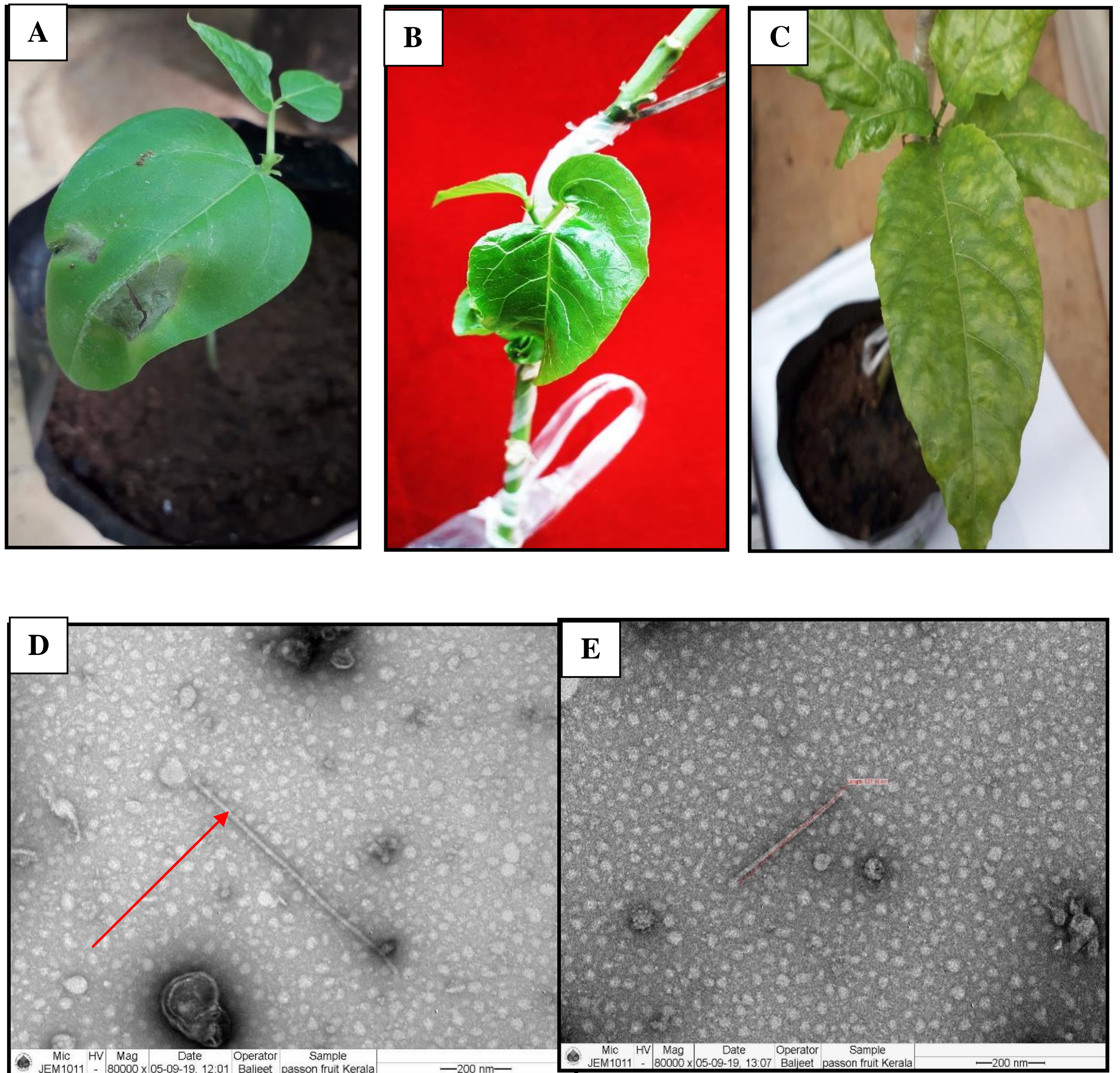


Plate 24: Characterization of virus associated with leaf malformation (VKALM)

A. Sap transmission – necrotic lesions

B. Graft transmission – malformed leaves on root stock

C. Graft transmission – symptom appearance on newly emerged leaves of root stock

D. Transmission electron microscopic image – long flexuous rods of virus particles

E. Size of virus – 527.36 nm

on cowpea seedlings through sap transmission produced typical local lesions confirming the viral etiology of disease.

4.5.3.2 Morphological Characterisation

Electron microscopy was done by leaf dip method (Bhat *et al*., 2004) using JEM1011 electron microscope at Advanced Centre of Plant Virology, I.A.R.I, New Delhi. Leaf dip samples were prepared using negative stain, two per cent uranyl acetate (pH-4.5) from infected passion fruit leaves. Typical flexuous rods of virus were observed in the electron micrograph (Plate 24). The size of the virus was 527.36 nm. Based on these observations, it was confirmed that the pathogen associated with leaf malformation disease in passion fruit is *Poty virus* belonging to the family *Potyviridae*.

Based on pathogenicity studies, cultural, morphological, biochemical and biological characterisation the pathogens associated with various symptoms were identified up to genus level and summarised in Table 4.9.

4.6 MOLECULAR CHARACTERIZATION

Molecular characterization of the fungal, bacterial and viral pathogens was carried out for the species level identification, confirmation of species and molecular diagnosis respectively. It was carried out by genomic DNA isolation and amplification of specific region using PCR which was done at the Molecular Lab for Plant Disease Diagnosis, Department of Plant Pathology, College of Horticulture, Vellanikkara and Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram. The retrieved sequences were analysed by using the BLASTn programme of NCBI to find the nucleotide homology and species level identification.

4.6.1 Fungal pathogens

The isolates of fungal pathogens collected from different surveyed locations were characterised up to the generic level as *Alternaria* sp., *Rhizoctonia* sp., *Lasiodiplodia* sp., *Sclerotium* sp. and *Colletotrichum* sp. based on cultural and morphological characterisation. Molecular characterization of these fungal isolates for the species level identification was done by genomic DNA isolation and amplification of ITS region of the fungal genome. Characterization of *Alternaria* sp. and *Rhizoctonia*

Table 4.10: Identification of pathogens associated with different symptoms

Sl. No.	Designated code	Disease	Associated Pathogen
1.	VKALS	Leaf spot	*Colletotrichum* sp.
2.	MDKLS	Leaf spot	*Alternaria* sp.
3.	MNPLS	Leaf spot	*Lasiodiplodia* sp.
4.	NELLS	Leaf spot	*Colletotrichum* sp.
5.	NENLS	Leaf spot	*Colletotrichum* sp.
6.	MVPLS	Leaf spot	*Colletotrichum* sp.
7.	AMBLS	Leaf spot	*Colletotrichum* sp.
8.	THNLS	Leaf spot	*Colletotrichum* sp.
9.	MVPLB	Leaf blight	*Rhizoctonia* sp.
10.	NENLB	Leaf blight	*Rhizoctonia* sp.
11.	MDKFR	Fruit rot	*Alternaria* sp.
12.	NELFR	Fruit rot	*Colletotrichum* sp.
13.	MVPFR	Fruit rot	*Colletotrichum* sp.
14.	PAZFR	Fruit rot	*Sclerotium* sp.
15.	MNPWt	Wilt	*Ralstonia* sp.
16.	KANWt	Wilt	*Ralstonia* sp.
17.	VKALM	Leaf malformation	*Poty virus*

sp. was carried out in the Molecular Lab for Plant Disease Diagnosis, Department of Plant Pathology, College of Horticulture, Vellanikkara. The remaining were outsourced at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram.

4.6.1.1 Alternaria sp.

The pathogen associated with the fruit rot (MDKFR) and leaf spot (MDKLS) was confirmed as *Alternaria* sp. based on cultural and morphological characterisation. For the species level identification of *Alternaria* sp, genomic DNA isolation was carried out by CTAB method followed by ITS sequencing.

4.6.1.1.a Quality and quantity of genomic DNA

The concentration of isolated DNA was quantified using Nanodrop Spectrophotometer. The quantity of isolated DNA was recorded as 1290 µg/ml with an absorbance ratio of 2.01 ($A_{260/280}$). Electrophoresis was carried out using 0.8 per cent agarose gel in 1X TAE buffer as electrophoresis buffer at 80 V for one hour. Gel documentation of the total DNA was done in Univech Cambridge gel documentation system (Plate 25).

4.6.1.1.b Standardisation of Annealing temperature

The annealing temperature was standardised for the amplification of ITS region by using Vertiflex program in PCR Thermo Cycler (Eppendof Mastercycler) with temperatures 50, 50.2, 50.7, 51.5, 52.5, 53.5, 54.5, 55.5, 56.5, 57.3, 57.8, 58.0° C. The amplification was obtained at 51.5 and 52.5° C (Plate 27)

Table 4.11: Standardisation of annealing temperature of *Alternaria* sp.

Temperature	Result	Temperature	Result
50° C	Faint bands	54.5° C	Good bands
50.2° C	Faint bands	55.5° C	Good bands
50.7° C	Faint bands	56.5° C	Faint bands
51.5° C	Good bands	57.3° C	Faint bands
52.5° C	Good bands	57.8° C	Faint bands
53.5° C	Good bands	58.0° C	Faint bands

4.6.1.1.c Sequencing

The PCR product having the amplified ITS region of *Alternaria* sp. was sent to Agri genome, Kochi for purification and sequencing. The nucleotide sequence of MDKLS retrieved was given in Fig. 2a.

4.6.1.1.d In silico analysis

The nucleotide sequence of ITS region of *Alternaria* sp. retrieved from Agri genome, Kochi were analysed using nucleotide BLASTn programme and the output showed sequence similarities with the reported ITS gene sequences of *Alternaria porri.* The sequence retrieved showed a similarity of 99.82 per cent with 100 per cent query coverage and maximum score of 1050 with *Alternaria porri* accessions; MK905449.1 and KU504325.1 (Fig. 3a)

4.6.1.1.e Phylogenetic analysis

The evolutionary relationship between the *Alternaria porri* isolate (from MDKLS) and other accessions from the top hits of NCBI blast was carried out by Mega X software. The analysis revealed that the isolate from the leaf spot sample, MDKLS showed maximum evolutionary relationship with the accession HM131982 (Fig. 3a)

4.6.1.2 Rhizoctonia sp.

Based on cultural and morphological characters, the pathogen associated with MVPLB and NENLB was confirmed as *Rhizoctonia* sp. The species level identification was carried out by genomic DNA isolation using CTAB method and ITS sequencing.

4.6.1.2.a Quality and quantity of genomic DNA

Total DNA concentration was quantified as 2300 µg/ml with an absorbance ratio of 2.04 ($A_{260/280}$). The purity of isolated DNA was checked by electrophoresis using 0.8 per cent agarose gel in 1X TAE buffer as electrophoresis buffer at 80 V for one hour and gel documentation of the total DNA was done in Univech Cambridge gel documentation system (Plate 25)

4.6.1.2.b Standardisation of Annealing temperature

The annealing temperature was standardised for the amplification of ITS region

by using Vertiflex program in PCR Thermo Cycler (Eppendof Mastercycler) with temperatures 52.8, 52.9, 50.7, 53.3, 53.5, 53.7, 53.9, 54.1, 54.3, and 54.4° C. The amplification was obtained at 53.7 and 53.9° C (Plate 26).

Table 4.12 Standardisation of annealing temperature of *Rhizoctonia* sp.

Temperature	Result	Temperature	Result
52.8° C	No amplification	53.7° C	Good bands
52.9° C	No amplification	53.9° C	Good bands
53.1° C	No amplification	54.1° C	No amplification
53.3° C	No amplification	54.3° C	No amplification
53.5° C	No amplification	54.4° C	No amplification

4.6.1.2.c Sequencing

The amplified PCR product of the ITS region of *Rhizoctonia* sp. was sent to Agri genome, Kochi for purification and sequencing. The nucleotide sequence retrieved was given in Fig. 2b.

4.6.1.2.d In silico analysis

The nucleotide sequence of ITS region of *Rhizoctonia* sp. retrieved from Agri genome, Kochi were analysed using nucleotide BLAST and it showed sequence similarities with the reported ITS gene sequences of *Rhizoctonia solani.* The isolate showed similarity (99.73%) with 100 per cent query coverage and maximum score of 1347 with the *Rhizoctonia solani* accession-HG934415.and a similarity (99.59%) with 99 per cent query coverage and maximum score of 1339 with HG934419, the accession of the same. (Fig. 3b)

The remaining isolates were sent to Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram for ITS sequencing and the sequences retrieved were analysed using nucleotide BLAST and sequence homology was identified.

4.6.1.2.e ***Phylogenetic analysis***

The evolutionary relationship between the *Rhizoctonia solani* isolate (from

NENLB) and other accessions from the top hits of NCBI blast was carried out by Mega X software. The analysis revealed that the isolate showed maximum evolutionary relationship with the accession HG934415 (Fig. 3b).

4.6.1.3 Lasiodiplodia sp.

Cultural and morphological characters revealed that the pathogen associated with the leaf spot (MNPLS) as *Lasiodiplodia* sp. The nucleotide sequence retrieved from RGCB for *Lasiodiplodia* sp. (Fig. 2c) was analysed by comparing with the other nucleotide sequences available in NCBI and it revealed that there is a sequence similarity (94.72%) with 64 per cent query coverage and a maximum score of 1046 with accession LC074359.1 of *Lasiodiplodia theobromae*. The sequence also showed a similarity (91.49%) with accession (JX282407) of the same. Other accessions with similarity are MT497430.1 (91.23%), KX965601.1 (94.4%), MK530029.1 (97.91%) and MK530038.1 (97.90%). The evolutionary relationship between the *Lasiodiplodia theobromae* isolate (from MNPLS) and other accessions from the top hits of NCBI blast was carried out by Mega X software. The analysis revealed that the isolate from the leaf spot sample, MNPLS showed maximum evolutionary relationship with the accession MT103322 (Fig. 3c).

4.6.1.4 Sclerotium sp.

Based on cultural and morphological characters, the pathogen associated with the fruit rot sample (PAZFR) collected from Pazhayannur was confirmed as *Sclerotium* sp. at the generic level. Several hits obtained by the sequence comparison of *Sclerotium* sp. (PAZFR) retrieved from RGCB (Fig. 2d) with other sequences of NCBI showed a sequence similarity of 98.28 with accessions of *Athellia rolfsii* (MN872304), 98.14 (MN622806), 98.54 (MN610008) and 98.54 (MN610006) per cent with query coverage of 65, 66, 64 and 64 per cent respectively. The sequence also showed similarity of more than 98 per cent with other accessions like MN610004, MN610002, MN610000, MK424482 and MH542664 (Fig. 2d). The evolutionary relationship between the *Sclerotium rolfsii* isolate (from PAZFR) and other accessions from the top hits of NCBI blast was carried out by Mega X software. The analysis revealed that the isolate from the leaf spot sample, PAZFR showed maximum evolutionary relationship with the

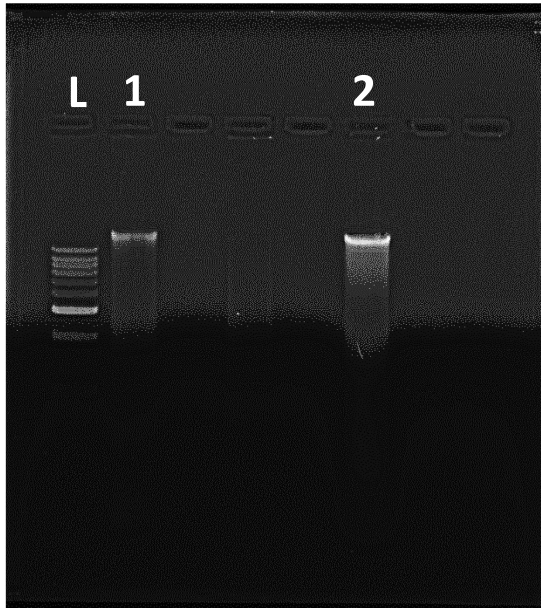


Plate 25: Gel profile of isolated DNA of fungal isolates

L- 1 kb ladder, 1- Genomic DNA of *Alternaria* sp., 2- Genomic DNA of *Rhizoctonia* sp.

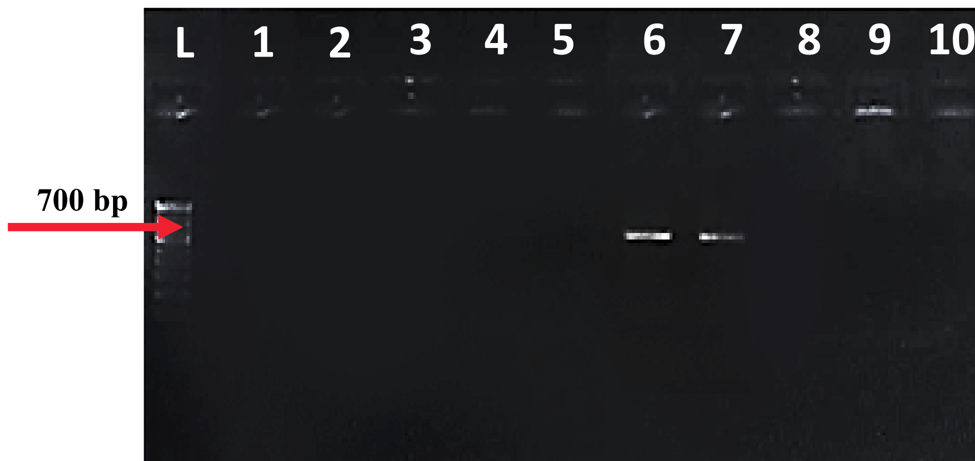


Plate 26: Standardisation of annealing temperature of *Rhizoctonia* sp.

L- 100 BP ladder

1- 52.8° C	6 - 53.7° C
2- 52.9° C	7 - 53.9° C
3- 53.1° C	8 - 54.1° C
4- 53.3° C	9 - 54.3° C
5- 53.5° C	10- 54.4° C

accession MN610004 (Fig. 3d).

4.6.1.5 Phomopsis sp.

The cultural and morphological studies revealed that the pathogen associated with the bud blight sample (KLYBB) collected from Kolazhy, Thrissur was *Phomopsis* sp. *In silico* analysis of the sequence retrieved showed that the sequence has a similarity of 99.50 per cent with the sequence of *Diaporthe phaseolorum* (Accession no. JQ514150) confirming the pathogen as *Diaporthe phaseolorum.* The evolutionary relationship between the isolate (from KLYBB) and other accessions from the top hits of NCBI blast was carried out by Mega X software. The analysis revealed that the isolate from the bud blight sample, KLYBB showed maximum evolutionary relationship with the accession HM012819 (Fig. 3e).

4.6.1.6 Colletotrichum sp.

The pathogen associated with VELLS, NELLS, NELFR, NENLS, MVPLS, MVPFR, THNLS, and AMBLS were identified at the generic level as *Collettotrichum* sp. This fungus was isolated from the leaf spots and fruit rot samples. The pathogen was isolated from all the surveyed districts. Hence, it can be considered as the major fungal pathogen in passion fruit. Therefore, sequencing of ITS region of all the *Colletotrichum* isolates was carried out for the diversity and phylogenetic analysis. The results of *in silico* analysis of sequences all *Colletotrichum* isolates were given below.

Isolate NELLS:

The comparison of nucleotide sequence of *Colletotrichum* sp. (Fig. 2e) isolated from NELLS, the leaf spot samples of Nelliyampathy with the sequences available in NCBI revealed that the sequence showed a similarity (of 96.84%) with three accessions of *Colletotrichum brevisporum* namely KX463009, KU662423 and KP734236 with 53 per cent query maximum score of 739 for each. It also showed similarity (of 96.61%) with other accessions of same pathogen namely MT606208, MT560321, MT361074, MT319074, MT232983 and MT012108 with 53 per cent query coverage (Fig. 3e).

5’TTCTGTAGGTGAACCTGCGGAGGGATCATTACACAAATATGAAGGCGGGCTG
GAATCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCTTGTCTTTTGCGTACT
TCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACATAAACCTTTTGTA
ATTGCAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCAACAACGGATCT
CTTGGTTCTGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAGTGTGAATT
GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATT
CCAAAGGGCATGCCTGTTCGAGCGTCATTTGTACCCTCAAGCTTTGCTTGGTGT
TGGGCGTCTTGTCTCTAGCTTTGCTGGAGACTCGCCTTAAAGTAATTGGCAGCC
GGCCTACTGGTTTCGGAGCGCAGCACAAGTCGCACTCTCTATCAGCAAAGGTCT
AGCATCCATTAAGCCTTTTTTCAACTTTTGACCTCGGATCAGGTAGGGATACCC
GCTGAACTTAAGCATATCAATAAGCGGAGGAA 3’

Fig 1 a: Nucleotide Sequence of ITS region of *Alternaria* sp.

5’TCCTCCGCTTATTGATATGCTTAAGTTCAGCGGGTAGTCCTACCTGATTTGAG
ATCAGATCATAAAATTAATATTTGTCCAAGTCAATGGACTGTTAGAAGCAGTTC
ATCTGCATTTACCTTGGCCACCCCTTTTTAACAGGGTGTCCTCAGCGATAGATA
ACTTATCACGCTGAGTGGAACCAAGCATAACACTGAGATCCAGCTAATGCACA
AAGAGGAGCAGGTGTGAAGCTGCAATAGACCTCCAATACCAAAGCAGAACCA
ATTGAGTTAACAAAAGGTTTGACTTTGAAGATCGCATGATACTCAAACAGGCAT
GCTCCAAGGAATACCAAGGAGCGCAAGGTGCGTTCAAAGATTCGATGATTCAC
TGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCG
AGAGCCAAGAGATCCGTTGTTGAAACTTAGTATTAGATGTGTTACATCCATTAC
ATTCATTTTAAAATAAATTGAGTTTATATAGATTAAGTAGACAGAAGTCCAAGG
AGAGTAGGTCCAATAAAGTTCCTTCCCCCCTAGAAAACATCTGTCTCACAGGTG
CACAGGTGTGTATGGGATGAAAGAGAAAGGTGTGCACATGCCCCCAAGTTAAT
TAGGGGCCAGCTACAACCAAACTCTACATTAATTCAATAATGATCCTTCCGCAG
GTTCACCTACGGAAACCTTGTTACGACTTTTACTTCCA 3’

Fig 1 b: Nucleotide Sequence of ITS region of *Rhizoctonia* sp.

Sequences producing significant alignments Download ˅ Manage Columns ˅ Show 100

select all *99 sequences selected* GenBank Graphics Distance tree of results

	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	Alternaria porri isolate VakAp-4 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	1050	1050	100%	0.0	99.82%	MK905449.1
☑	Alternaria sp. BAB-5554 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	1050	1050	100%	0.0	99.82%	KU504325.1
☑	Alternaria sp. voucher HQU PS16 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA ge	1048	1048	99%	0.0	99.82%	MK640581.1
☑	Alternaria porri isolate PeeAp-11 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gen	1048	1048	99%	0.0	99.82%	MK905450.1
☑	Alternaria brassicicola strain SWTPF17 18S ribosomal RNA gene, partial sequence	1048	1048	99%	0.0	99.82%	MG840692.1
☑	Alternaria sp. BAB-5088 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	1048	1048	99%	0.0	100.00%	KT186141.1
☑	Alternaria sp. GA-F4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transc	1048	1048	99%	0.0	99.82%	KF888649.1
☑	Alternaria sp. AS4 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcrib	1048	1048	99%	0.0	99.82%	KF888647.1
☑	Alternaria brassicicola isolate Ab4UP 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, ar	1048	1048	99%	0.0	99.82%	KF542552.1
☑	Uncultured Alternaria clone FM1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and int	1048	1048	99%	0.0	99.82%	JX564139.1
☑	Alternaria sp. IA249 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcri	1048	1048	99%	0.0	99.82%	AY154699.1
☑	Alternaria sp. IA248 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene and internal transcri	1048	1048	99%	0.0	99.82%	AY154698.1
☑	Alternaria sp. isolate LD18L-14-sp(2) small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA	1046	1046	99%	0.0	99.82%	MN955458.1
☑	Alternaria brassicicola isolate MVR1 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA	1046	1046	99%	0.0	99.82%	MK158222.1
☑	Alternaria longipes strain PV1603 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and ir	1046	1046	99%	0.0	99.82%	KY027029.1
☑	Alternaria sp. BAB-5097 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal tra	1046	1046	99%	0.0	100.00%	KT186151.1
☑	Alternaria aff. longipes RLC16.3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and int	1046	1046	99%	0.0	99.82%	DQ156337.1

Fig 2a: BLASTn text output of nucleotide sequence of ITS region of *Alternaria* sp.

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	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	Rhizoctonia solani genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene,	1347	1347	100%	0.0	99.73%	HG934415.1
☑	Rhizoctonia solani genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene,	1339	1339	99%	0.0	99.59%	HG934419.1
☑	Rhizoctonia solani strain JWC-22-P1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,	1328	1328	99%	0.0	99.32%	KJ866422.1
☑	Rhizoctonia solani genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene,	1328	1328	99%	0.0	99.32%	HG934417.1
☑	Rhizoctonia solani isolate RT 14-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8	1328	1328	99%	0.0	99.32%	FJ746938.1
☑	Rhizoctonia solani isolate RT 7-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S	1328	1328	99%	0.0	99.32%	FJ746912.1
☑	Thanatephorus cucumeris isolate Str44 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	1328	1328	99%	0.0	99.32%	DQ102447.1
☑	Rhizoctonia solani genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene,	1325	1325	100%	0.0	99.18%	HG934418.1
☑	Rhizoctonia solani genomic DNA containing 18S rRNA gene, ITS1, 5.8S rRNA gene, ITS2 and 28S rRNA gene,	1325	1325	99%	0.0	99.18%	HG934416.1
☑	Rhizoctonia solani strain JWC-22-P2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,	1323	1323	99%	0.0	99.18%	KJ866423.1
☑	Rhizoctonia solani strain JWC-4-P2 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5	1323	1323	99%	0.0	99.18%	KJ866419.1
☑	Rhizoctonia solani isolate RT 14-3 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8	1323	1323	99%	0.0	99.18%	FJ746940.1

Fig 2b: BLASTn text output of nucleotide sequence of ITS region of *Rhizoctonia* sp.

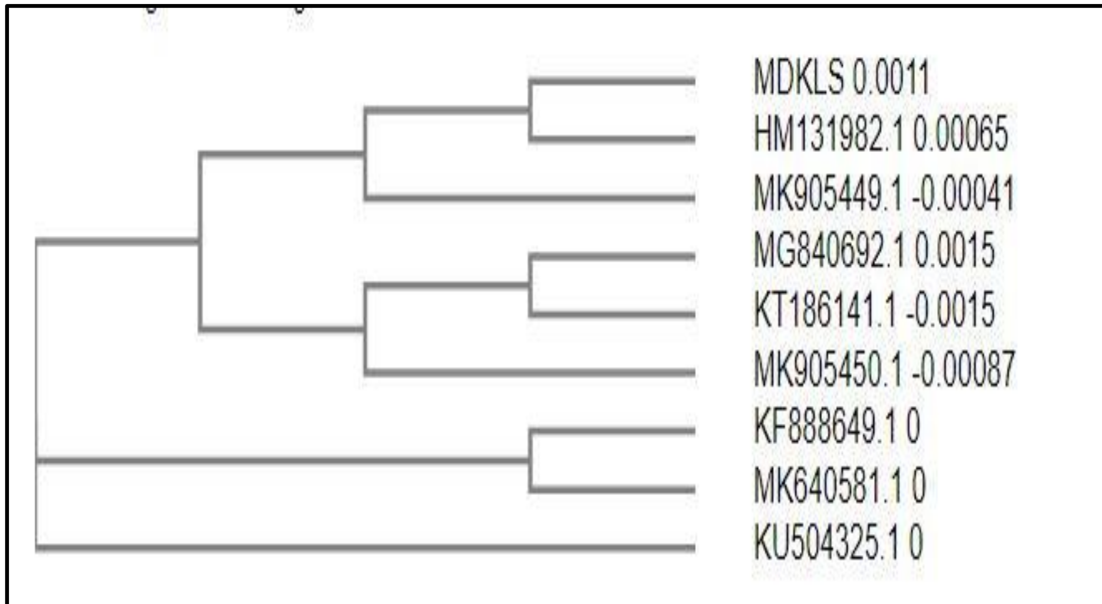

Fig 3 a: Phylogenetic analysis of *Alternaria porri*

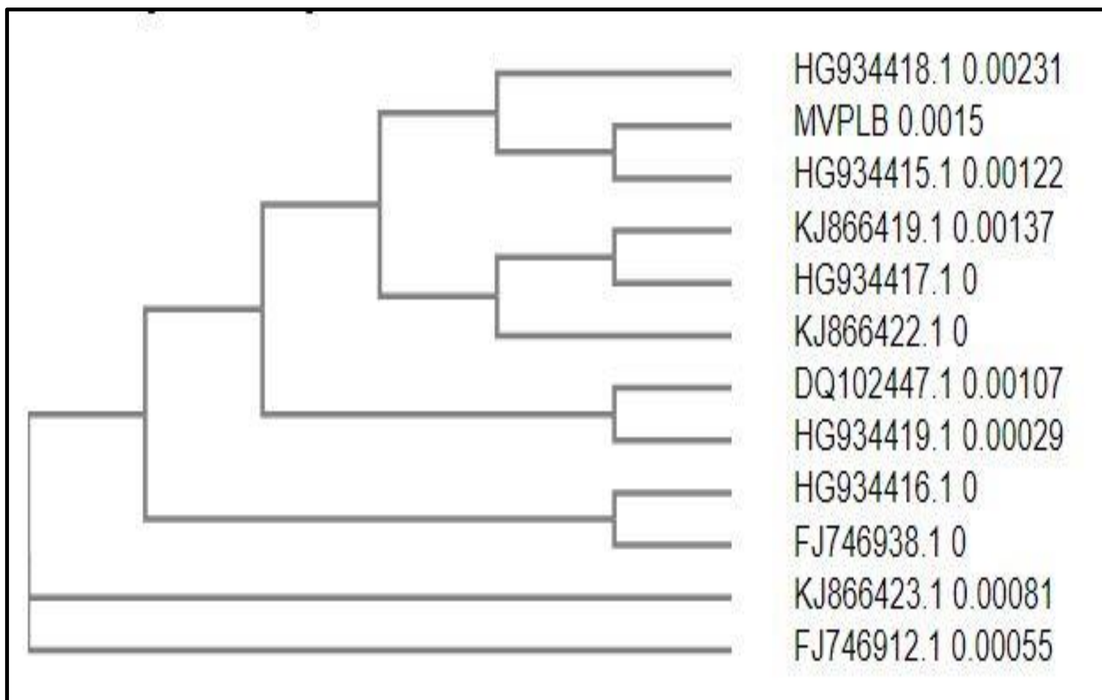

Fig 3 b: Phylogenetic analysis of *Rhizoctonia solani*

5’TAGATCAACGCTACGGCAACTGGTCTTCGGCTTCCGATGAAGACTCTCCCA
TTCCCAAGCAGGCTAGTGTATAGCCTGGACGGCATGCCCCCGATACAAGGC
AATTGCGTTCAAAGATCGTGATGATGATTCTGCAATGCACATTACTATGGCA
TGAGTGCGTTCTTCATCGATGCCAGAATCCAAAGTCCGTGTGAAAGTTTTAG
TTTATTAAACTGTTTATCAGAGTCTGCGTTTACGACGAGTTGAAGTCCTTGC
GCGAAGCCGCCAAACGCAAGAGAGGTACGTTCACAACGGTGAGAGTCGAG
CCGAGCTCGATAAAGCTCGTAATGATCGCTCTGTTGAGACTTGCGGAAGGAT
CATTACCGAGTTTTCGAGCTCCGGCTTGACTCTCCCACCCTTTGTGAACGTAC
CTCTGTTGCTTTGGCGGCTCCGGCCGCCAAAGGACCTTCAAACTCCAGTCAG
TAAACGCAGACGTCTGATAAACAAGTTAATAAACTAAAACTTTCAACAACG
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAA
TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC
CCTTGGTATTCCGGGGGGCATGCCTGTTCGAGCGTCATTACAACCCTCAAGC
TCTGCTTGGAATTGGGCACCGTCCTCACTGCGGACGCGCCTCAAAGACCTCG
GCGGTGGCTGTTCAGCCCTCAAGCGTAGTAGAATACACCTCGCTTTGGAGCG
GTTGGCGTCGCCCGCCGGACGAACCTTCTGAACTTTTCTCAAGGTTGACCTC
GGATCAGGTAGGGATACCCGCTGAACTTAAGCATATCAATAAGCGAGCGAA
TATTACGAGCGGAGGAGCTCAGCTCTAACTCGCAGGAGCGAACCTACCTCG
CGTGACGATGGCGGCTCCGCCCGAAGGACTATCAACTCTTCGCTAAACGCA
GACTTCGATTAACAGGTTAAATAAAACTAAACTTTCACACGGACTTGTTTCT
GGCTTCATGAAAAAGCACAATGGCATAGTAATGTGAAATTGCATAATCCTG
AATCATGCAACCTTGAAGCATTGGGCTTCGTATTCCGGGGGGCCATGCTGTC
AAGCGTCATCAACCTAGCTGCTGAAATTGACTCTGCTAGACCCGCATCAGAC
GGTCT3’

Fig 1c: Nucleotide Sequence of ITS region of *Lasiodiplodia* sp.

5’CATATTTATACCAAGGGTCTCAGTTGAATCCTCTAGGTAACCTAGCCATTAG
AGTTAGACTAGCGTTCAGTATGTTCAGATTCTTTCCTCGAGCCTACAGTTGTTC
ACTACGGTGCACTGTTTAAAGAATAGCTTCTCACATGTGGCATCATGCATATT
GTTTCTATCCAGCACAACTACCTGGTCCATCTGAATTACAATTAATGATCCCTT
TCCTTTCCGTAGGTGAACATGCGGAAGGATCATTATTGAATTCATATATGCAA
AGGAGTTGTGCTGGTAATAAATATTGCATGTGCACACTCTGAAGCTATATAAT
ATATACACCTGTGAACCAACTGTAGTCTGGAGAAATCCTGACTATGATTACTC
TATATAACTCTTATTGTATGTTACATAGAACGATCTCATATTGAAGCTTTGTTT
TTTTTTACAAGTTTCTCTTAATTGAAAAATACACAACTTTCAACAACGGATCTC
TTGGCTCTTGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAAT
TGCAGAATCCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCCCTTTGGTA
TTCCGAGGGGCATGCCTGTTTGAGAGTCATTAAATTCTCAACCTTACAAATTT
TTGTATTTGTCAAGGCTTGGATGTGAGAGTTGCTAGTTAAGAATATCTGACTG
GCTCTCTTTAAAACTATTAGTAGGACATATAGAAATGCCTGCGGTTGGTGTGA
TAATATGTCTACGCCTATACCAAAGGGGATTCTAGCTTGTATGCACTACTTAT
AAAATCATGCGCATATATCTAGCAATATAAGTGCAATATATTGACCATTTGAC
CTCAAATCAAGGTAGGACTACCCCGCCTGAAACTTAAGCATAGTCATATAAAT
CGGGAGGAAGAAAGGGAGATCCATTATATCTTGGTAATTGTCGGAATAGGGT
AGCCAAATGAGTACATTGACATGGCCTGGGCAAAGAATAGACCAGTATCGCA
TGAATGCCACGACACATCGGGTGAGAACACACCTAATTCTCAGTTAGT3’

Fig 1d: Nucleotide Sequence of ITS region of *Sclerotium* sp.

5'TGGGAAGTAAAAAATCGTAACAAGGTCTCCGTTGGTGAACCAGCGGAGG
GATCATTGCTGGAACGCGCTTCGGCGCACCCAGAAACCCTTTGTGAACTTA
TACCTATCTGTTGCCTCGGCGCAGGCCGGCCTCTTCACTGAGGCCCCCTGG
AAACAGGGAGCAGCCCGCCGGCGGCCAACTAAACTCTTGTTTCTATAGTG
AATCTCTGAGTAAAAAACATAAATGAATCAAAACTTTCAACAACGGATCT
CTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTG
AATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCTC
TGGTATTCCGGAGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCCT
GGCTTGGTGATGGGGCACTGCCTTCTAACGAGGGCAGGCCCTGAAATCTA
GTGGCGAGCTCGCTAGGACCCCGAGCGTAGTAGTTATATCTCGTTCTGGAA
GGCCCTGGCGGTGCCCTGCCGTTAAACCCCCAACTTCTGAAAATTTGACCT
CGGATCAGGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGGAGG
AAAAAA3'

Fig 1e: Nucleotide Sequence of ITS region of *Phomopsis* sp.

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Diaporthe phaseolorum isolate FM1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, a	1098	1098	98%	0.0	99.50%	JQ514150.1
Phomopsis sp. xy22 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and inte	1081	1081	98%	0.0	99.01%	KX397027.1
Phomopsis sp. BPEF83 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal t	1081	1081	98%	0.0	99.01%	KF219920.1
Diaporthe phaseolorum strain E99382 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	1081	1081	98%	0.0	99.01%	AY577815.1
Diaporthe sp. SAB-2009a strain Q1983 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gen	1077	1077	99%	0.0	98.84%	FJ799938.1
Diaporthe phaseolorum 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal t	1074	1074	96%	0.0	99.66%	HM012819.1
Fungal sp. ARIZ B126.p 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal t	1074	1074	96%	0.0	99.49%	FJ612924.1
Diaporthe phaseolorum isolate WAA02 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene	1070	1070	98%	0.0	98.68%	KT964567.1
Diaporthe phaseolorum isolate LF13 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RN	1066	1066	94%	0.0	100.00%	KX510129.1
Phomopsis sp. CML 1535 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and interna	1066	1066	95%	0.0	99.66%	JN153053.1
Diaporthe phaseolorum strain H206 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, a	1064	1064	94%	0.0	100.00%	KX020564.1
Diaporthe phaseolorum strain pssp5 internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete s	1064	1064	98%	0.0	98.51%	AF001020.2
Diaporthe ueckerae isolate DPFT33 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RN	1062	1062	94%	0.0	100.00%	MK111104.1
Diaporthe phaseolorum isolate 8.1.1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, a	1062	1062	98%	0.0	98.51%	KP133195.1
Diaporthe ueckerae isolate DPFT27 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RN	1059	1059	93%	0.0	100.00%	MK111102.1

Fig 2e: BLASTn text output of nucleotide sequence of ITS region of *Phomopsis* sp.

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	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	Lasiodiplodia theobromae genes for 18S rRNA, ITS1, 5.8S rRNA, ITS2, 28S rRNA, partial and complete sequer	1046	1403	64%	0.0	94.72%	LC074359.1
☑	Lasiodiplodia theobromae isolate Aravind Mdu UI2 18S ribosomal RNA gene, partial sequence; internal transcril	1042	1042	65%	0.0	91.49%	JX282407.1
☑	Lasiodiplodia theobromae isolate SS_32.1 small subunit ribosomal RNA gene, partial sequence; internal transcı	1031	1031	65%	0.0	91.23%	MT497430.1
☑	Lasiodiplodia theobromae isolate KhL-Giza internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA	1005	1233	54%	0.0	94.40%	KX965601.1
☑	Lasiodiplodia theobromae isolate BPPCA144 small subunit ribosomal RNA gene, partial sequence; internal tran	985	1237	64%	0.0	97.91%	MK530029.1
☑	Lasiodiplodia theobromae isolate BPPCA167 small subunit ribosomal RNA gene, partial sequence; internal tran	983	1235	64%	0.0	97.90%	MK530038.1
☑	Lasiodiplodia theobromae isolate BPPCA134 small subunit ribosomal RNA gene, partial sequence; internal tran	979	979	47%	0.0	97.89%	MK530023.1
☑	Lasiodiplodia theobromae strain CP/VPC-2 small subunit ribosomal RNA gene, partial sequence; internal transc	977	1237	64%	0.0	97.89%	MT103322.1
☑	Lasiodiplodia theobromae isolate BPPCA136 small subunit ribosomal RNA gene, partial sequence; internal tran	977	977	47%	0.0	97.89%	MK530024.1
☑	Lasiodiplodia theobromae isolate MRR-142 small subunit ribosomal RNA gene, partial sequence; internal transc	972	972	52%	0.0	94.79%	MT075444.1
☑	Lasiodiplodia theobromae isolate BPPCA160 small subunit ribosomal RNA gene, partial sequence; internal tran	972	972	47%	0.0	97.72%	MK530033.1
☑	Lasiodiplodia theobromae isolate BPPCA117 small subunit ribosomal RNA gene, partial sequence; internal tran	970	1230	64%	0.0	97.39%	MK530016.1

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Fig 2c: BLASTn text output of nucleotide sequence of ITS region of *Lasiodiplodia* sp.

Sequences producing significant alignments Download Manage Columns Show 100

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	Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
☑	Athelia rolfsii isolate Kale078 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1	1210	1210	65%	0.0	98.28%	MN872304.1
☑	Athelia rolfsii isolate BX small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8	1208	1208	66%	0.0	98.14%	MN622806.1
☑	Athelia rolfsii isolate MSB5-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1	1203	1203	64%	0.0	98.54%	MN610008.1
☑	Athelia rolfsii isolate MSB4-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1	1203	1203	64%	0.0	98.54%	MN610006.1
☑	Athelia rolfsii isolate MSB3-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1	1203	1203	64%	0.0	98.54%	MN610004.1
☑	Athelia rolfsii isolate MSB2-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1	1203	1203	64%	0.0	98.54%	MN610002.1
☑	Athelia rolfsii isolate MSB1-2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1	1203	1203	64%	0.0	98.54%	MN610000.1
☑	Athelia rolfsii isolate YG2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.	1203	1203	65%	0.0	98.13%	MK424482.1
☑	Athelia rolfsii isolate Aconitum carmichaelii Debx small subunit ribosomal RNA gene, partial sequence; internal t	1203	1203	64%	0.0	98.68%	MH542664.1
☑	Athelia rolfsii isolate Tr_Stevia AR-02 small subunit ribosomal RNA gene, partial sequence; internal transcribed	1201	1201	64%	0.0	98.54%	MK288124.1
☑	Sclerotium delphinii strain CBS 221.46 small subunit ribosomal RNA gene, partial sequence; internal transcribe	1201	1201	64%	0.0	98.68%	MH856168.1
☑	Athelia rolfsii strain CBA-HW06 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S	1201	1201	65%	0.0	97.99%	GU567776.1

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Fig 2d: BLASTn text output of nucleotide sequence of ITS region of *Sclerotium* sp.

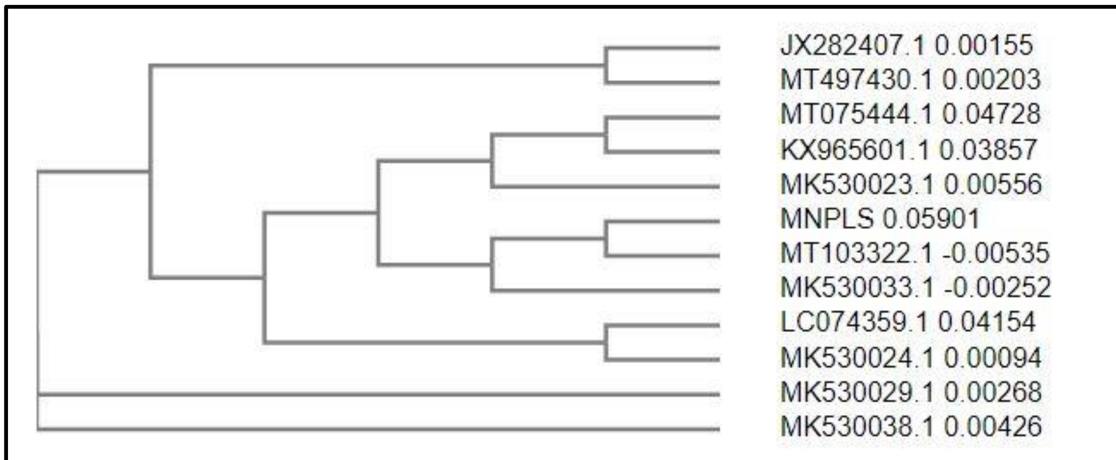

Fig 3 c: Phylogenetic analysis of *Lasiodiplodia theobromae*

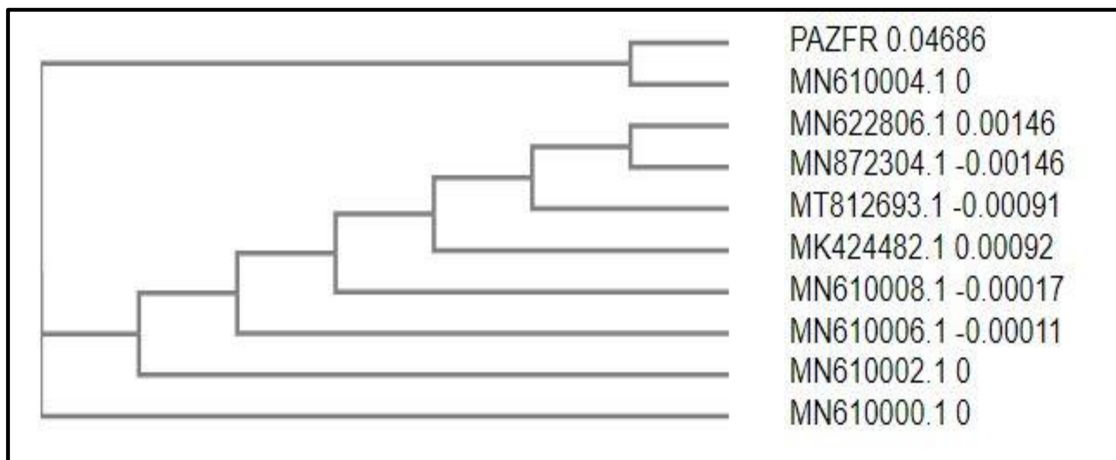

Fig 3 d: Phylogenetic analysis of *Sclerotium rolfsii*

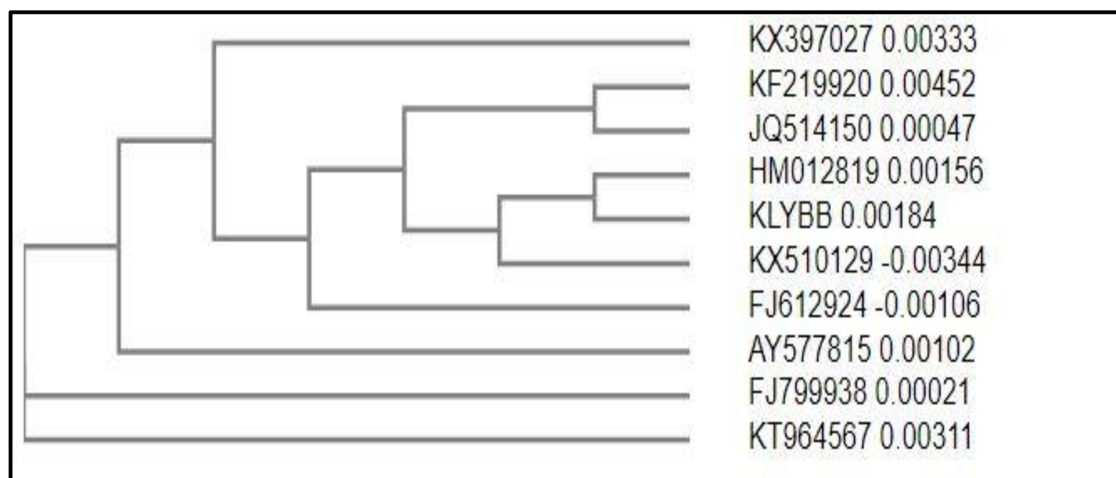

Fig 3 e: Phylogenetic analysis of *Diaporthe phaseolorum*

Isolate NELFR:

The sequence comparison of NELFR collected from the fruit rot sample of Nelliyampathy with the available sequences in the NCBI database showed that the sequence of NELFR has similarity of cent per cent and a query per cent of 62 with the accessions of *Colletotrichum brevisporum i.e.* MT560321, MT012108, MT232983, MH063256, MG525134, MG525133, KY986893, JN943068, JN943071. It also showed a similarity (99.78%) and 62 per cent query with other accessions of the same pathogen namely MT361074, MH883805 and MK330019.

Isolate MVPLS:

The nucleotide sequence of ITS region of MVPLS collected from leaf spot sample of Muvattupuzha of Ernakulam district was subjected to NCBI BLASTn and the comparison with the available sequences in the database revealed 98.92 per cent similarity with a query coverage of 60 per cent with the accessions of *Colletotrichum brevisporum* namely MT012108, MH063256 and JN687987. The sequence also showed a similarity of 99.13 per cent with the accessions; MT626673, MT569150, MN305797, MN153755, MK569226, MK569225, MK530194 and MK530193.

Isolate MVPFR:

The nucleotide sequence of ITS region of the isolate-MVPFR collected from the fruit rot sample was analysed by sequence comparison with the available sequences of NCBI database using NCBI BLASTn and the results revealed that the sequence have a similarity of 97.53 per cent with a query per cent of 74 per cent with *Colletotrichum brevisporum* accessions, MT012108 and MH063256. The sequence also showed homology with other accessions of *C. brevisporum* with a similarity of 97.7 per cent are MT569150, MN305797, MK569227, MK569225, MK530194, MK530193, MG525134 and MG525133.

Isolate VELLS:

The sequence of ITS region retrieved for the isolate VELLS collected from the leaf spot samples of Vellanikkara, Thrissur district was subjected to NCBI BLAST. The comparison of nucleotide sequence of ITS showed 97.72 per cent similarity with a

query coverage of 58 per cent with the ITS region of *Colletotrichuim brevisporum* belonging to the accessions MT560321. It also showed a similarity of 99.82 per cent with a query coverage of 54 per cent with other two accessions of same pathogen *i.e.* KC815123 and HM163177.

Isolate AMBLS:

Several hits obtained by the sequence comparison of ITS region of the isolate AMBLS collected from Ambalavayal of Wayanad showed a similarity of 99.57 per cent with a query coverage of 56 per cent with *Colletotrichum brevisporum* accessions namely MT560321, MT012108, MG461529, KT949407, KR534660, KC815123, GU066694, GU066653 and FJ233190.

Isolate THNLS:

The sequence comparison of ITS region of the isolate-THNLS collected from Thankamani of Idukki district showed a similarity of 100 per cent with a query coverage of 62 per cent with *Colletotrichum brevisporum* belonging to the accessions MT560321, MT012108, MT232983, MH063256, MG525134, MG525133, KY98689, JN943068, JN943071 and MT361074.

Isolate NENLS:

The sequence of ITS region of NENLS, the isolate of leaf spot collected from Nemmara region of Palakkad was analysed by NCBI BLASTn. The sequence retrieved for the isolate showed a similarity of 99.40 per cent with *Colletotrichum queenslandicum* belonging to the accessions MN547511, MK119210 and MF380919.

To sum up the molecular characterisation of *Colletotrichum* isolates, the seven isolates (VELLS, NELLS, NELFR, MVPLS, MVPFR, THNLS, and AMBLS) was *Colletotrichum brevisporum* and one isolate (NENLS) was *Colletotrichum queenslandicum.*

5’TCAACATCCGGCACCTCGTCCGCCAAGATCTCGTGCGGGCCCCCAAT
GGGCTTTACAAAGACTTGCGTGAATTCACCTGATTTAGCCAATGGACCA
CTTAACTAACGCACATCTGGCTGCTTTTTAATGAAGCGCAGAAACCCC
AAAAAGTCTCGTTGTTAAAAAGTTTTGATTAATTCGCTGCAACCACCTC
CAGGAGGGAAGGTCGTTGCTCAAAAAAGTTTATTGTTCTCCGCCGCGG
CGGTGGGGCCGCCCAAGAGGGTTCCCGCGCCGGAGCTGGCTCCGGCTG
CCCGCCGAGGCAAACGTTGAGGTATTGTTCACCAAAGGGTTATAGAGC
GTAACTCGATAATGATTCCCCTCTTTACGGAGTGAACTTCAGTGAGTTG
CCGCTCCTTACAACCCTTCGGGTAACACACCTCTAACCTTGCCTTTGGTT
AGCAGCCGGACCCCAGCTCCGGCGCCCGGAGCCGCCTTCTCGGCGCGC
CCCACCCGCCGGCGGACCACTAAACTCTATTGCAACGACGTCTCTTCTG
AGTGGTACAAGCAAATAATCAAAACTTTTAACAACGGATCTCTTGGTTC
TGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGC
AGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGC
ATTCTGGCGGGCATGCCTGTTCCAGCGTCATTTCAACCCTCAAGCACCG
CTTGGCGTTGGGGCCCTACGGCTTCCGTAGGCCCCGAAATACAGTGGC
GGACCCTCCCGGAGCCTCCTTTGCGTAGTAACATACCACCTCGCACT 3’

Fig 1f: Nucleotide Sequence of ITS region of isolate NELLS

5'CCATTGCTAAAGGTTGGTTTCATGATGCCAATTACCTTTAGCTTAGCA
ATTAGCGTGCTTTTTTATGCAGGCAGATAAAGGTCCGTGTTGAAAGTGT
GATAATTTGCCTGACACACTCTCGAGAGGAGAGTGCGTTGCCAAAGAG
TTATGTTCCGCCCGGCGGGTGGGGGCGGCCAGAGGCGCTTCCGGCCGA
AGCTGGCTCGGCTGCCCGCCGGGCAAACGTTGAGGTATGTTCACAAAG
GTTATAGGAGCGTAACTCGATCATGTATGGCCAACCTGCGGAGGGATC
ATTATCGAGTTACCGCTCCTTATAACCCTTTGTGAACATACCTCAAACG
TTGCCTCGGCGGGCAGCCGGAGCCCAGCTCCGGCGCCCGGAGCCGCCT
TCTCGGCGCGCCCCACCCGCCGGCGGACCACTAAACTCTATTGCAACG
ACGTCTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTTTAACAACG
GATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAG
TAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACA
TTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCA
ACCCTCAAGCACCGCTTGGCGTTGGGGCCCTACGGCTTCCGTAGGCCC
CGAAATACAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTAGTAACAT
ACCACCTCGCA 3'

Fig 1g: Nucleotide Sequence of ITS region of isolate NELFR

5’GCCTGAAAACGACATCGTCCGCCAGATGGCGCCAATGCTCAAGGTGC
TGATCATGACTTTGCAATTAGCATCATCTATGCATTCTGCTTTCTTTCGA
TGAGGGCAGAAGCCCAATGCTTCTGTTTTGAAATGTTTCGATCAGTTTC
TTTATCCTGCTCAGTGGCGAGTAGTGCTATGCTCCTCTGCTTGCTGATA
TGCTCCTACGCGTACTGATGTGCTCCTCCGCTTATGGATACGCTCCTCC
GCTTATTGATATGCTCCTCCGCTTATTGATATGCTCCTCCGCTTATTGAT
ACTCTCCTACTGATATGCTTAAGTTCAGCGGGTATTCCTACCTGATCCG
AGGTCAACCTTGATAAATTGGGGGGTTTTACGGCAGGAGTCCCTCCGG
ATCCCAGTGCGAGGTGGTATGTTACTACGCAAAGGAGGCTCCGGGAGG
GTCCGCCACTGTATTTCGGGGCCTACGGAAGCCGTAGGGCCCCAACGC
CAAGCGGTGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCGCC
AGAATGCTGGCGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTG
AATTCTGCAATTCACATTATCTCGGCGCGACCAACCCGCCGGCGGACC
ACTAAACTCTATTGCAACGACGTCTCTTCTGAGTGGTACAAGCAAATA
ATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAA
CGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCA
TCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATG
CCTGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGGCGTTGGGGCC
CTACGGCTTCCGTAGGCCCCGAAATACAGTGGCGGACCCTCCCGGAGC
CTCCTTTGCGTAGTAACATACCC3’

Fig 1h: Nucleotide Sequence of ITS region of isolate MVPFR

5’ACTTGCTAGTTCCCTCATAATCCTTCGTGAACCTCCCTCAAAAGTTGCCTCG
GTGGGCAGCCGGAGCCCAGCTCCGGCGCCCGGAGCCGCCTTCTCGGCGCGAC
CAACCCGCCGGCGGACCACTAAACTCTATTGCAACGACGTCTCTTCTGAGTGG
TACAAGCAAATAATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCC
TGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGGCGTTGGGGCCCTACGG
CTTCCGTAGGCCCCGAAATACAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGT
AATAACATACCACCTCGCACTGGGATCCGGAGGGACTCCTGCCGTAAAACCC
CCCAATTTATCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAC
CATATCAGTAGAGAGAATCATTAAGCGGAGGAGCATATCAATAAGCGGAGGA
GTATATCATAAGCGGAGGAGGATATCCGTAACCGGAGGAGCACCGGAATCCC
CTCGTGGGAACCCACCCCACGAGGGCCGAAGGACCTTATGGAAAAACTCCCG
TGGAGAGTTGGTCAATAGGAT3’

Fig 1i: Nucleotide Sequence of ITS region of isolate MVPLS

5’CCATTGCTAAAGGTTGGTTTCATGATGCCAATTACCTTTAGCTTAGCAATTA
GCGTGCTTTTTTATGCAGGCAGATAAAGGTCCGTGTTGAAAGTGTGATAATTT
GCCTGACACACTCTCGAGAGGAGAGTGCGTTGCCAAAGAGTTATGTTCCGCC
CGGCGGGTGGGGGCGGCCAGAGGCGCTTCCGGCCGAAGCTGGCTCGGCTGCC
CGCCGGGCAAACGTTGAGGTATGTTCACAAAGGTTATAGGAGCGTAACTCGA
TCATGTATGGCCAACCTGCGGAGGGATCATTATCGAGTTACCGCTCCTTATAA
CCCTTTGTGAACATACCTCAAACGTTGCCTCGGCGGGCAGCCGGAGCCCAGCT
CCGGCGCCCGGAGCCGCCTTCTCGGCGCGCCCCACCCGCCGGCGGACCACTA
AACTCTATTGCAACGACGTCTCTTCTGAGTGGTACAAGCAAATAATCAAAACT
TTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCAC
ATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACC
CTCAAGCACCGCTTGGCGTTGGGGCCCTACGGCTTCCGTAGGCCCCGAAATAC
AGTGGCGGACCCTCCCGGAGCCTCCTTTGCGTA GTAACATACCACCTCGCA3’

Fig 1j: Nucleotide Sequence of ITS region of isolate THNLS

5’AGGCAAGCTGCTGGAAAGGCTTACACTGCGCGATGGCGCCATGCTAAGTTG
TTTCATGATGCAATCACTACCTAGGCAGTCGTGCTTATCGAAGCCAGAAAACA
AAGTCCCGTTGTTAAAAAGTTTGTTAATTGCTTGTACCCACTCAGAAGGAAGC
GTGCAAAGAAGTTAATGTTCTGCCGCGGGGTGGCGCGCCAGAGCGCTCCCGC
GCGAGCTGGGCTCGGCTGCCCGCCGGGCAACGTTGAGTATGTTCACAGAGGG
TATATGAGCGGTAACTCGATATTGATCCTCGCTAACGTGCGGAGGGATCATTA
TCGAGTTACCGCTCCTTATAACCCTTTGTGAACATACCTCAAACGTTGCCTCG
GCGGGCAGCCGGAGCCCAGCTCCGGCGCCCGGAGCCGCCTTCTCGGCGCGCC
CCACCCGCCGGCGGACCACTAAACTCTATTGCAACGACGTCTCTTCTGAGTGG
TACAAGCAAATAATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGA
TGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAA
TCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCC
TGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGGCGTTGGGGCCCTACGG
CTTCCGTAGGCCCCGAAATACAGTGGCGGACCCTCCCGGAGCCTCCTTTGCGT
AGTAACATACCACCTCGCACTGGGATCCGGAGGGACTCCTGCCGTAAAACCC
CCCAATTTATCAAGGTTGACCTCGGATCAGGTAGGAATACCCGCTGAACTTAA
GCATATCAATAAGCGGAAGAATATCGAGTTAGGCTCCTTATAACCCTTTGTGG
AAAATACCTCAACGTTGCCGCGGCGGGCAGCCCGAGCCCAGCTTCAGCGCCG
GAACCCCTTCTACGGCCCCCCCACCGGAAGAAAATAAAACTTTAGCAGCAC
CATTCCTCCTGAGGTGGGTACGGCGAAATAAA3’

Fig 1k: Nucleotide Sequence of ITS region of isolate VELLS

5’CTCCCGCCCCCCCGGGCGGGTCGGCGCCCGCCGGAGGATAACCAAACTCTG
ATTTAACGACGTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTTTAACA
ACGGATCTCTTGGTTCTGATCGATGAAGAACGCAGCGAAATGCGATAAGTAA
TGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCC
GCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTC
TGCTTGGTGTTGGGGCCCTACAGCCGATGTAGGCCCTCAAAGGTAGTGGCGG
ACCCTCCGGGACCCTCAATAAGCGGTGAAGCATATCAATACGCGGAGGAGCA
TATCAATAAGCGGAGGAGCATATCAATAAGCGGAGGAGCATATCAATAAGCG
AGAGCATGCAT3’

Fig 1l: Nucleotide Sequence of ITS region of isolate NENLS

5'ATGATTCGCTTACGACTAGCCAGCCATGCTGCTGGAATAGGCCTCGAAAG
CATGCCGCCGAATGGCGCGCCAATTGCTCAAGTTGCTGATTCATGATTGCAA
TCACATTACCTACCGCATGCGTGCTTCTTGTGCAGGCCAGAAACCCAAAGTC
CGTTGTTAAAGTTTTGATTAATTTGGCTGTAACCCACCTCAGTAGGGAGTCG
TTGCAAAGAAGTTTATGTTCCGCCGGGGGGTGGGTCGCCGAGAAGCGCTCC
CGGCGCTGGAGCTGGCTCGGGCTGCCCGCCGCGGCAACGTTGAGGTATGTT
CACAAAGGGTTTATGAGCCGTAACTCGATAATGATCCCGCTAACCTGTGGA
GGGATCATTATCGAGTTACCGCTCTTTATAACCCTTTGTGAACATACCTCAA
ACGTTGCCTCGGCGGGCAGCCGGAGCCCAGCTCCGGCGCCCGGAACCGCCT
TCTCGGCGCGCCCCACCCGCCGGCGGACCACTAAACTCTATTGCAACGACGT
CTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTTTAACAACGGATCTCTT
GGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT
GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGC
ATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTT
GGCGTTGGGGCCCTACGGCTTCCGTAGGCCCCGAAATACAGTGGCGGACCC
TCCCGGAGCCTCCTTTGCGTAGTAACATACCACCTCGCACTGGGATCCGGAG
GGACTCCTGCCGTAAAACCCCCCAATTTATCAAGGTTGACCTCGGATCAGGT
AGGAATACCCGCTGAACTTAAGCATATCAGTAAGACAAGAAGAAATAAGCG
GAGGAGCATATCAACAAGCGGAGGAATATATCAATATTGCGGAGGAGGGC
AGCCAGAACCAGGAGCAGCACGGCAACACCCTAGGGACCACGCCCCGGAG
AACATAAGCTCTATAGCACGACTTCTCTCTGAGGTTGGTTACAAGCCAAATA
ATCAAACTTTTAAACAACGAACCTTGGTTTCTGGCTCGAATCAAAAAAAGC
ACGAACTGCATAGGTAATGTGATTTGCAGAAGTCATGAATCAGCACTTTGA

Fig 1m: Nucleotide Sequence of ITS region of isolate AMBLS

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Colletotrichum brevisporum isolate Colletotrichum brevisporum A554 internal transcribed spacer 1, partial sequ	739	739	53%	0.0	96.84%	KX463009.1
Colletotrichum sp. G35 MS-2016 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and	739	739	53%	0.0	96.84%	KU662423.1
Colletotrichum cf. magnum G14 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1 and	739	739	53%	0.0	96.84%	KP734236.1
Colletotrichum sp. voucher MF2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and	734	734	53%	0.0	96.61%	MT606208.1
Colletotrichum brevisporum isolate jpsk3 small subunit ribosomal RNA gene, partial sequence; internal transcri	734	734	53%	0.0	96.61%	MT560321.1
Colletotrichum brevisporum strain GM1 small subunit ribosomal RNA gene, partial sequence; internal transcrib	734	734	53%	0.0	96.61%	MT361074.1
Colletotrichum liaoningense strain A10 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge	734	734	53%	0.0	96.61%	MT319074.1
Colletotrichum brevisporum isolate Cb internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge	734	734	53%	0.0	96.61%	MT232983.1
Colletotrichum brevisporum strain SN9 small subunit ribosomal RNA gene, partial sequence; internal transcribe	734	734	53%	0.0	96.61%	MT012108.1
Colletotrichum brevisporum isolate UACH289 small subunit ribosomal RNA gene, partial sequence; internal tra	734	734	53%	0.0	96.61%	MK862121.1
Colletotrichum gloeosporioides isolate Ang-ITS small subunit ribosomal RNA gene, partial sequence; internal tr	734	734	53%	0.0	96.61%	MN305797.1
Colletotrichum sp. strain FRZ4-4 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene an	734	734	53%	0.0	96.61%	MK399761.1

Fig 2f: BLASTn text output of nucleotide sequence of ITS region of NELLS

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Colletotrichum brevisporum isolate jpsk3 small subunit ribosomal RNA gene, partial sequence; internal transcr	852	852	62%	0.0	100.00%	MT560321.1
Colletotrichum brevisporum strain SN9 small subunit ribosomal RNA gene, partial sequence; internal transcrib	852	852	62%	0.0	100.00%	MT012108.1
Colletotrichum brevisporum isolate Cb internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ge	850	850	62%	0.0	100.00%	MT232983.1
Colletotrichum brevisporum strain MBG16102401 small subunit ribosomal RNA gene, partial sequence; intern	850	850	62%	0.0	100.00%	MH063256.1
Colletotrichum brevisporum strain COUFAL0170 small subunit ribosomal RNA gene, partial sequence; interna	850	850	62%	0.0	100.00%	MG525134.1
Colletotrichum brevisporum strain COUFAL0169 small subunit ribosomal RNA gene, partial sequence; interna	850	850	62%	0.0	100.00%	MG525133.1
Colletotrichum brevisporum isolate S38 18S ribosomal RNA gene, partial sequence; internal transcribed spac	850	850	62%	0.0	100.00%	KY986893.1
Glomerella cingulata var. brevispora strain LC0525 internal transcribed spacer 1, partial sequence; 5.8S ribos	850	850	62%	0.0	100.00%	JN943068.1
Glomerella cingulata var. brevispora strain LC0870 internal transcribed spacer 1, partial sequence; 5.8S ribos	848	848	62%	0.0	100.00%	JN943071.1
Colletotrichum brevisporum strain GM1 small subunit ribosomal RNA gene, partial sequence; internal transcrit	846	846	62%	0.0	99.78%	MT361074.1
Colletotrichum brevisporum strain TCHD 18S ribosomal RNA gene, partial sequence; internal transcribed spa	846	846	62%	0.0	99.78%	MH883805.1
Colletotrichum brevisporum clone EIPP 44 small subunit ribosomal RNA gene, partial sequence; internal trans	846	846	62%	0.0	99.78%	MK330019.1

Fig 2g: BLASTn text output of nucleotide sequence of ITS region of NELFR

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Colletotrichum brevisporum strain SN9 small subunit ribosomal RNA gene, partial sequence; internal transcribe	898	898	74%	0.0	97.53%	MT012108.1
Colletotrichum brevisporum strain MBG16102401 small subunit ribosomal RNA gene, partial sequence; interna	898	898	74%	0.0	97.53%	MH063256.1
Colletotrichum brevisporum isolate PECB0604 internal transcribed spacer 1, partial sequence; 5.8S ribosomal	896	896	74%	0.0	97.70%	MT569150.1
Colletotrichum gloeosporioides isolate Ang-ITS small subunit ribosomal RNA gene, partial sequence; internal tr	896	896	74%	0.0	97.70%	MN305797.1
Colletotrichum brevisporum strain YMTJ78 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN/	896	896	74%	0.0	97.70%	MK569227.1
Colletotrichum brevisporum strain YMTJ22 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN/	896	896	74%	0.0	97.70%	MK569225.1
Colletotrichum magnum isolate LP3.2 small subunit ribosomal RNA gene, partial sequence; internal transcribec	896	896	74%	0.0	97.70%	MK530194.1
Colletotrichum magnum isolate LP2.1 small subunit ribosomal RNA gene, partial sequence; internal transcribec	896	896	74%	0.0	97.70%	MK530193.1
Colletotrichum brevisporum strain COUFAL0170 small subunit ribosomal RNA gene, partial sequence; internal	896	896	74%	0.0	97.70%	MG525134.1
Colletotrichum brevisporum strain COUFAL0169 small subunit ribosomal RNA gene, partial sequence; internal	896	896	74%	0.0	97.70%	MG525133.1

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Fig 2h: BLASTn text output of nucleotide sequence of ITS region of MVPFR

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Colletotrichum brevisporum strain SN9 small subunit ribosomal RNA gene, partial sequence; internal transcribe	826	826	60%	0.0	98.92%	MT012108.1
Colletotrichum brevisporum strain MBG16102401 small subunit ribosomal RNA gene, partial sequence; interna	826	826	60%	0.0	98.92%	MH063256.1
Colletotrichum gloeosporioides 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S	826	826	60%	0.0	98.92%	JN687987.1
Colletotrichum brevisporum strain J1-1-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA (	824	824	60%	0.0	99.13%	MT626673.1
Colletotrichum brevisporum isolate PECB0604 internal transcribed spacer 1, partial sequence; 5.8S ribosomal I	824	824	60%	0.0	99.13%	MT569150.1
Colletotrichum gloeosporioides isolate Ang-ITS small subunit ribosomal RNA gene, partial sequence; internal tr	824	824	60%	0.0	99.13%	MN305797.1
Colletotrichum brevisporum voucher BP1569 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RI	824	824	60%	0.0	99.13%	MN153755.1
Colletotrichum brevisporum strain YMTJ78 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN/	824	824	60%	0.0	99.13%	MK569227.1
Colletotrichum brevisporum strain YMTJ58 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN/	824	824	60%	0.0	99.13%	MK569226.1
Colletotrichum brevisporum strain YMTJ22 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN/	824	824	60%	0.0	99.13%	MK569225.1
Colletotrichum magnum isolate LP3.2 small subunit ribosomal RNA gene, partial sequence; internal transcribed	824	824	60%	0.0	99.13%	MK530194.1
Colletotrichum magnum isolate LP2.1 small subunit ribosomal RNA gene, partial sequence; internal transcribed	824	824	60%	0.0	99.13%	MK530193.1

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Fig 2i: BLASTn text output of nucleotide sequence of ITS region of MVPLS

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Colletotrichum brevisporum strain J1-1-2 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA g	806	806	59%	0.0	98.27%	MT626673.1
Colletotrichum brevisporum isolate PECB0604 internal transcribed spacer 1, partial sequence; 5.8S ribosomal	806	806	59%	0.0	98.27%	MT569150.1
Colletotrichum brevisporum strain SN9 small subunit ribosomal RNA gene, partial sequence; internal transcribe	806	806	59%	0.0	98.27%	MT012108.1
Colletotrichum gloeosporioides isolate Ang-ITS small subunit ribosomal RNA gene, partial sequence; internal tr	806	806	59%	0.0	98.27%	MN305797.1
Colletotrichum brevisporum voucher BP1569 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RI	806	806	59%	0.0	98.27%	MN153755.1
Colletotrichum brevisporum strain YMTJ78 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN/	806	806	59%	0.0	98.27%	MK569227.1
Colletotrichum brevisporum strain YMTJ58 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN/	806	806	59%	0.0	98.27%	MK569226.1
Colletotrichum brevisporum strain YMTJ22 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN/	806	806	59%	0.0	98.27%	MK569225.1
Colletotrichum magnum isolate LP3.2 small subunit ribosomal RNA gene, partial sequence; internal transcribed	806	806	59%	0.0	98.27%	MK530194.1
Colletotrichum magnum isolate LP2.1 small subunit ribosomal RNA gene, partial sequence; internal transcribed	806	806	59%	0.0	98.27%	MK530193.1
Colletotrichum brevisporum strain MBG16102401 small subunit ribosomal RNA gene, partial sequence; interna	806	806	59%	0.0	98.27%	MH063256.1
Colletotrichum brevisporum strain CPDZ21 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN/	806	806	59%	0.0	98.27%	MK032210.1

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Fig 2j: BLASTn text output of nucleotide sequence of ITS region of THNLS

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Colletotrichum brevisporum isolate jpsk3 small subunit ribosomal RNA gene, partial sequence; internal transcr	1048	1048	58%	0.0	97.72%	MT560321.1
Glomerella magna strain LC-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S	1037	1037	54%	0.0	99.82%	KC815123.1
Glomerella magna strain CMM1642 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1	1035	1035	54%	0.0	99.82%	HM163177.1
Colletotrichum brevisporum strain YYGXZ07 18S ribosomal RNA gene, partial sequence; internal transcribed	1033	1033	54%	0.0	100.00%	KU319458.1
Colletotrichum magnum isolate LP2.1 small subunit ribosomal RNA gene, partial sequence; internal transcribe	1031	1031	54%	0.0	100.00%	MK530193.1
Glomerella magna strain CMM1734 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1	1031	1031	54%	0.0	100.00%	HM163187.1
Glomerella magna isolate CMM1822 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	1031	1031	54%	0.0	100.00%	HM015854.1
Glomerella magna strain CMM1824 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1	1029	1029	54%	0.0	99.65%	HM163193.1
Colletotrichum sp. 80CP/T 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene	1029	1029	54%	0.0	99.64%	GU066653.1
Colletotrichum magnum isolate LP3.2 small subunit ribosomal RNA gene, partial sequence; internal transcribe	1027	1027	53%	0.0	100.00%	MK530194.1
Colletotrichum brevisporum strain SN9 small subunit ribosomal RNA gene, partial sequence; internal transcrib	1026	1026	54%	0.0	99.82%	MT012108.1

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Fig 2k: BLASTn text output of nucleotide sequence of ITS region of VELLS

Sequences producing significant alignments Download Manage Columns Show 100

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Colletotrichum queenslandicum isolate ACSIKS_2101768 small subunit ribosomal RNA gene, partial sequence;	603	603	77%	3e-168	99.40%	MN547511.1
Colletotrichum queenslandicum isolate CS8 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RN	603	603	77%	3e-168	99.40%	MK119210.1
Colletotrichum sp. strain LDCMYE28 small subunit ribosomal RNA gene, partial sequence; internal transcribed	603	603	77%	3e-168	99.40%	MG980396.1
Colletotrichum sp. strain LDCMYE22 small subunit ribosomal RNA gene, partial sequence; internal transcribed	603	603	77%	3e-168	99.40%	MG980305.1
Colletotrichum sp. strain LDCMYE17 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene	603	603	77%	3e-168	99.40%	MG980286.1
Colletotrichum queenslandicum strain MEF77 small subunit ribosomal RNA gene, partial sequence; internal trai	603	603	77%	3e-168	99.40%	MF380919.1
Colletotrichum sp. isolate CR 14 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and	603	603	77%	3e-168	99.40%	KY659058.1
Colletotrichum sp. isolate CR 19 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and	603	603	77%	3e-168	99.40%	KY659055.1
Colletotrichum gloeosporioides strain KU-KP/RK/EF-24 internal transcribed spacer 1, partial sequence; 5.8S rib	603	603	77%	3e-168	99.40%	MG204864.1
Colletotrichum gloeosporioides strain Ps-36 18S ribosomal RNA gene, partial sequence; internal transcribed sp	603	603	77%	3e-168	99.40%	KU671363.1
Colletotrichum gloeosporioides isolate MTCC 10339 18S ribosomal RNA gene, partial sequence; internal transc	603	603	77%	3e-168	99.40%	KX099750.1
Colletotrichum sp. GRMP-58 internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA gene and inte	603	603	77%	3e-168	99.40%	KF516005.1

Fig 2l: BLASTn text output of nucleotide sequence of ITS region of NENLS

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Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Colletotrichum brevisporum isolate jpsk3 small subunit ribosomal RNA gene, partial sequence; internal transcri	841	841	56%	0.0	99.57%	MT560321.1
Colletotrichum brevisporum strain SN9 small subunit ribosomal RNA gene, partial sequence; internal transcribe	841	841	56%	0.0	99.57%	MT012108.1
Colletotrichum magnum strain Cmp9 small subunit ribosomal RNA gene, partial sequence; internal transcribed	841	841	56%	0.0	99.57%	MG461529.1
Colletotrichum magnum isolate Cmp6 18S ribosomal RNA gene, partial sequence; internal transcribed spacer	841	841	56%	0.0	99.57%	KT949407.1
Colletotrichum magnum voucher INBio:1362B internal transcribed spacer 1, partial sequence; 5.8S ribosomal F	841	841	56%	0.0	99.57%	KR534660.1
Glomerella magna strain LC-1 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S	841	841	56%	0.0	99.57%	KC815123.1
Colletotrichum sp. 132CP/S 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene	841	841	56%	0.0	99.57%	GU066694.1
Colletotrichum sp. 80CP/T 18S ribosomal RNA gene, internal transcribed spacer 1, 5.8S ribosomal RNA gene,	841	841	56%	0.0	99.57%	GU066653.1
Colletotrichum sp. EXMY-8 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribc	841	841	56%	0.0	99.57%	FJ233190.1
Colletotrichum brevisporum isolate Cb internal transcribed spacer 1, partial sequence; 5.8S ribosomal RNA ger	839	839	56%	0.0	99.57%	MT232983.1
Colletotrichum brevisporum strain MBG16102401 small subunit ribosomal RNA gene, partial sequence; interna	839	839	56%	0.0	99.57%	MH063256.1

Fig 2m: BLASTn text output of nucleotide sequence of ITS region of AMBLS

4.6.1.6.a Phylogenetic analysis

Out of the 14 isolates of the fungal pathogens collected, eight isolates (NELLS, NELFR, MVPFR, MVPLS, VELLS, THNLS, AMBLS and NENLS) belong to the same fungal genus *Colletotrichum*. Multiple sequence analysis was done with all these eight isolates by using the bioinformatics tool, ClustalW to find out the relationship among these isolates. Phylogenetic tree made to study the relationship between isolates (Fig. 4a) revealed that all these eight isolates shared a common ancestor.

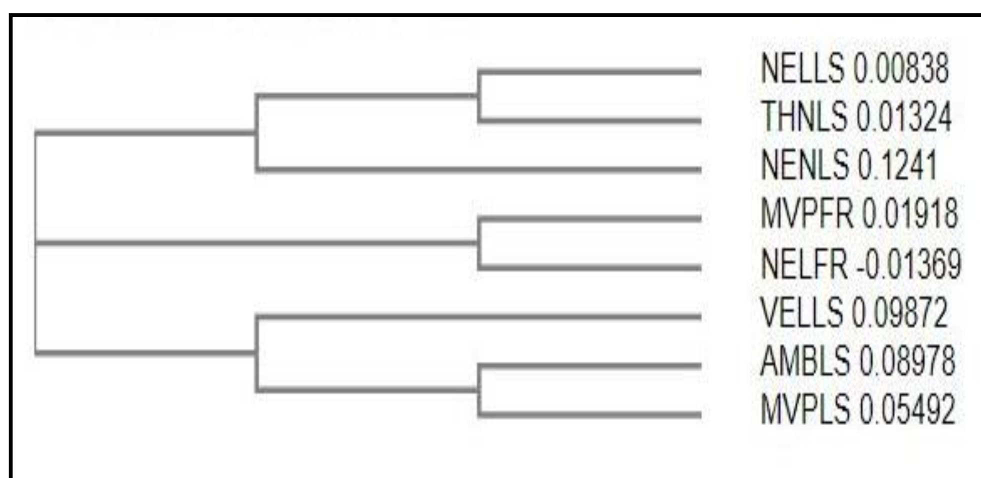


Fig. 3f: Phylogenetic tree of isolates collected belonging to the genus *Colletotrichum*

The *Colletotrichum* isolates collected from passion fruit formed three groups indicating a lineage with diversification. The isolates NELLS, THNLS, NENLS formed a single group and the fruit rot isolates NELFR and MVPFR formed a second group and VELLS, AMBLS and MVPLS formed a third group indicating some diversity within species based on the agro ecological situation and tissue in which the pathogen was infecting. *C. queenslandicum*, the causal agent of NENLS showed more similarity with NELLS and THNLS and placed as a subgroup in the group having these isolates.

A neighbouring-joining phylogenetic tree was made (Fig. 4b) with seven isolates (NELLS, MVPLS, AMBLS, THNLS, VELLS, MVPFR an NELFR) of *Colletotrichum brevisporum* collected from the study and other ITS sequences of *Colletotrichum* (six *C. brevisporum* accessions, four *C. capsici* and four *C. gloeosporoides*) reported from India and elsewhere available in the GeneBank

database to study the relationship between different species (Species diversification) belonging to the same genus *Colletotrichum.*

The neighbour-joining tree revealed that all the sequences were related, formed from same lineage and shared a common ancestor. The collected isolates of *Colletotrichum brevisporum* and the accessions of *Colletotrichum brevisporum* from the top hits of BLASTn output formed a single group. The accessions of *Colletotrichum gloeosporoides* and *Colletotrichum capsici* from Kerala retrieved from NCBI database formed sub groups of each in a second group and *C. queenslandicum* showed more similarity with *C. gloeosporoides* and was placed in their group. These two groups shared a common ancestor.

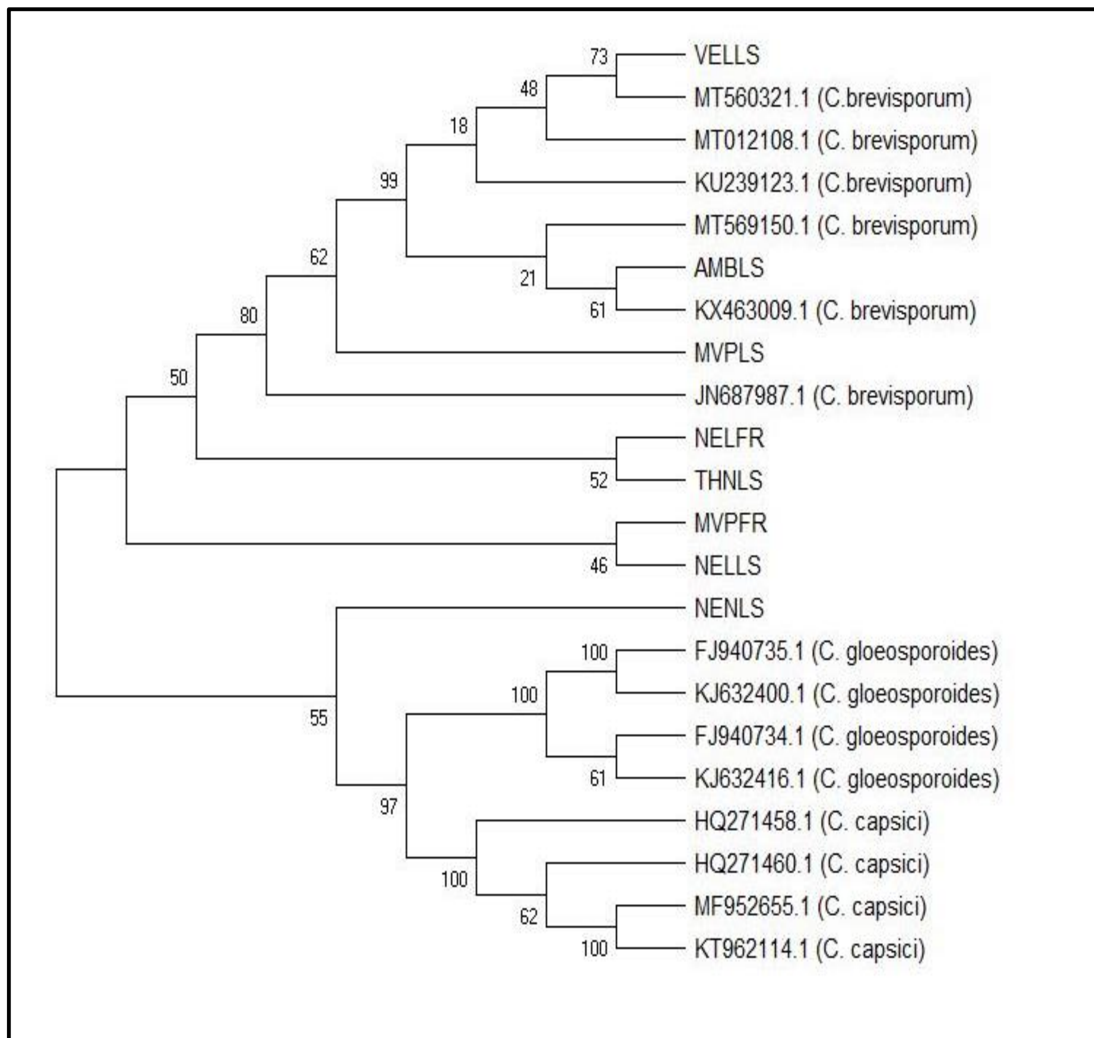


Fig. 3g: Phylogenetic tree showing relationship between species within the genus *Colletotrichum*

4.6.1.6.b DNA Barcoding

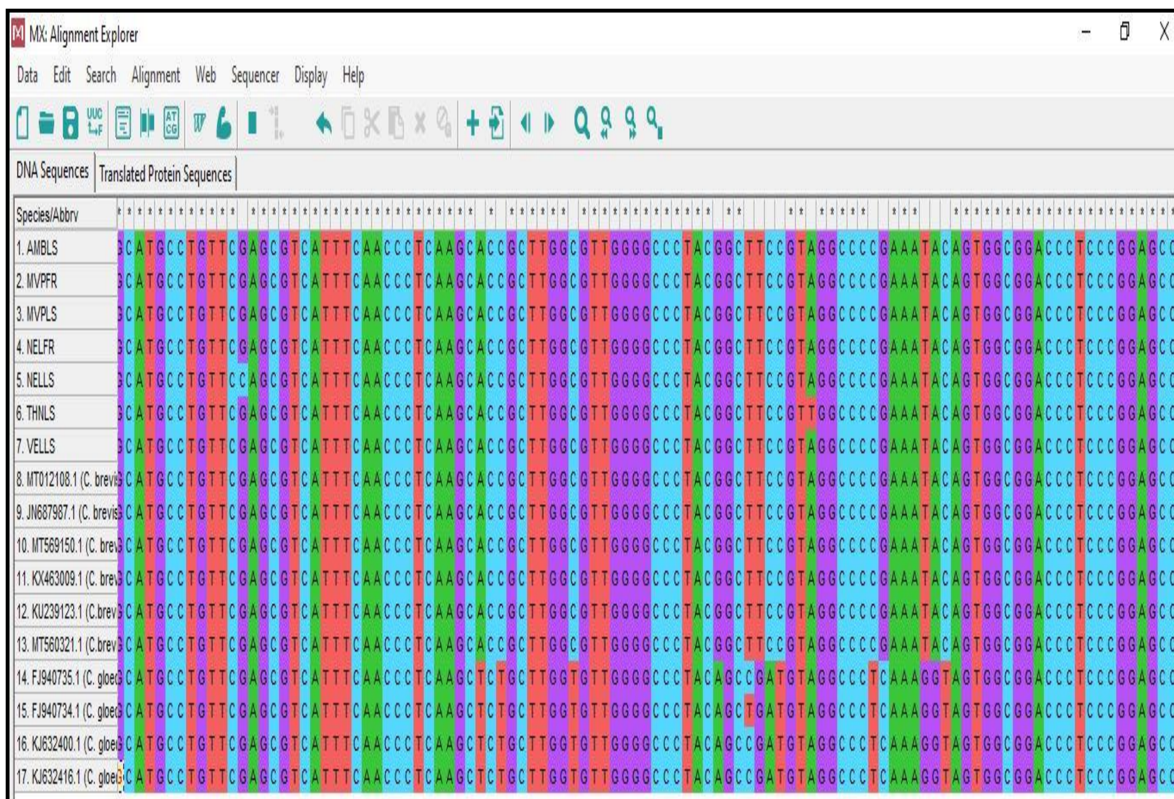
DNA barcoding of *C. brevisporum* isolates was done to confirm the identity of these isolates and barcodes was developed for *C. brevisporum* and *Colletotrichum gloeosporoides* to differentiate themby analysisng the gaps between the sequences. The sequences of local leaf spot isolates of *C. brevisporum* collected during the survey were multiple aligned with that of *C. brevisporum* isolates and *C. gloeosporoides* isolates retrieved from NCBI database using ClustalW tool provided by MEGA-X software (Fig. 4a and 4b). A set of 400 nucleotide bases was found to be aligned by the multiple alignment tool in which barcodes were only available in the first 100 bases and last 100 bases of the aligned sequences. Two hundred nucleotide bases in the middle were identical for both *C. brevisporum* and *C. gloeosporoides* isolates and a set of 32 nucleotide bases were identified to be specific for *C. brevisporum* and which is given in Fig. 5.

Similarly, barcodes were developed to differentiate *Colletotrichum queenslandicum* from *C. brevisporum.* A set of 31 nucleotide bases (Fig. 7) were identified from the multiple aligned sequence output from ClustalW (Fig. 6a and 6b). These nucleotides were specific for the *C. queenslandicum* isolates and was present in the first 100 bases and last 100 bases of the aligned sequence file. Hence these barcode gaps can be utilised to differentiate *C. queenslandicum* from *C. brevisporum.*

4.6.1.7Deposition of sequences

The sequences of ITS region of the fungal pathogens characterized were submitted in the database of Genebank. The accession numbers retrieved for the sequences were given the appendix VI.

Fig. 4a: Multiple Aligned sequences of *C. brevisporum* and *C. gloeosporoides*



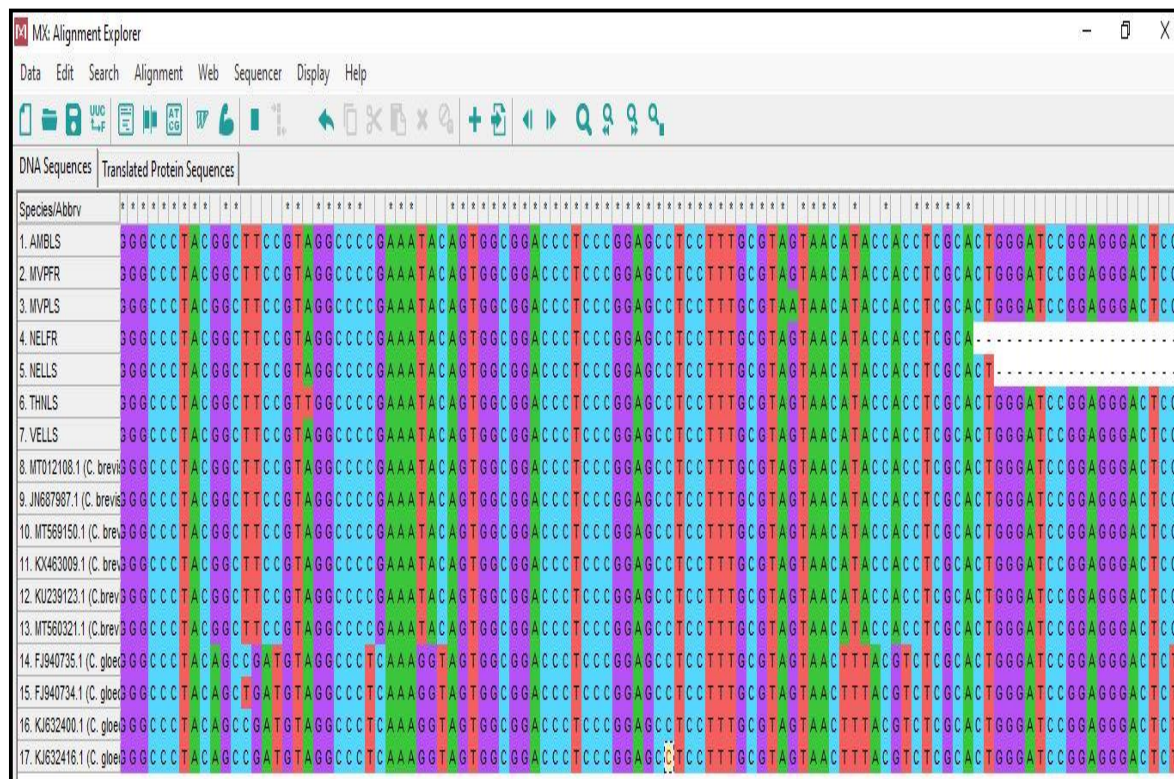


Fig. 4b: Multiple Aligned sequences of *C. brevisporum* and *C. gloeosporoides*

Fig. 5: Barcodes to differentiate *C. brevisporum* and *C. gloeosporoides*

Group	Isolates	460	462	472	475	476	478	479	484	504	505	508	516	519	520	529	728	730	737	751	755	756	757	766	767	771	772	773	812	814	815	817	818
Collected isolates	AMBLS	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
	MVPFR	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
	MVPLS	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
	NELFR	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
	NELLS	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
	THNLS	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
	VELLS	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
C. brevisporum	MT012108	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
	JN687987	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
	MT569150	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
	KX463009	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
	KU239123	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
	MT560321	G	G	G	T	T	T	C	C	C	C	T	A	G	C	C	A	C	C	G	T	C	C	C	G	T	A	C	A	A	C	A	C
C. gloeosporoides	FJ940735	C	C	C	C	C	C	G	G	T	A	C	G	T	T	T	T	T	T	A	G	A	T	T	C	G	G	T	T	T	A	G	T
	FJ940734	C	C	C	C	C	C	G	G	T	A	C	G	T	T	T	T	T	T	A	G	A	T	T	C	G	G	T	T	T	A	G	T
	KJ632400	C	C	C	C	C	C	G	G	T	A	C	G	T	T	T	T	T	T	A	G	A	T	T	C	G	G	T	T	T	A	G	T
	KJ632416	C	C	C	C	C	C	G	G	T	A	C	G	T	T	T	T	T	T	A	G	A	T	T	C	G	G	T	T	T	A	G	T

Fig 6a: Multiple Aligned sequences of *C. queenslandicum* and *C. brevisporum*

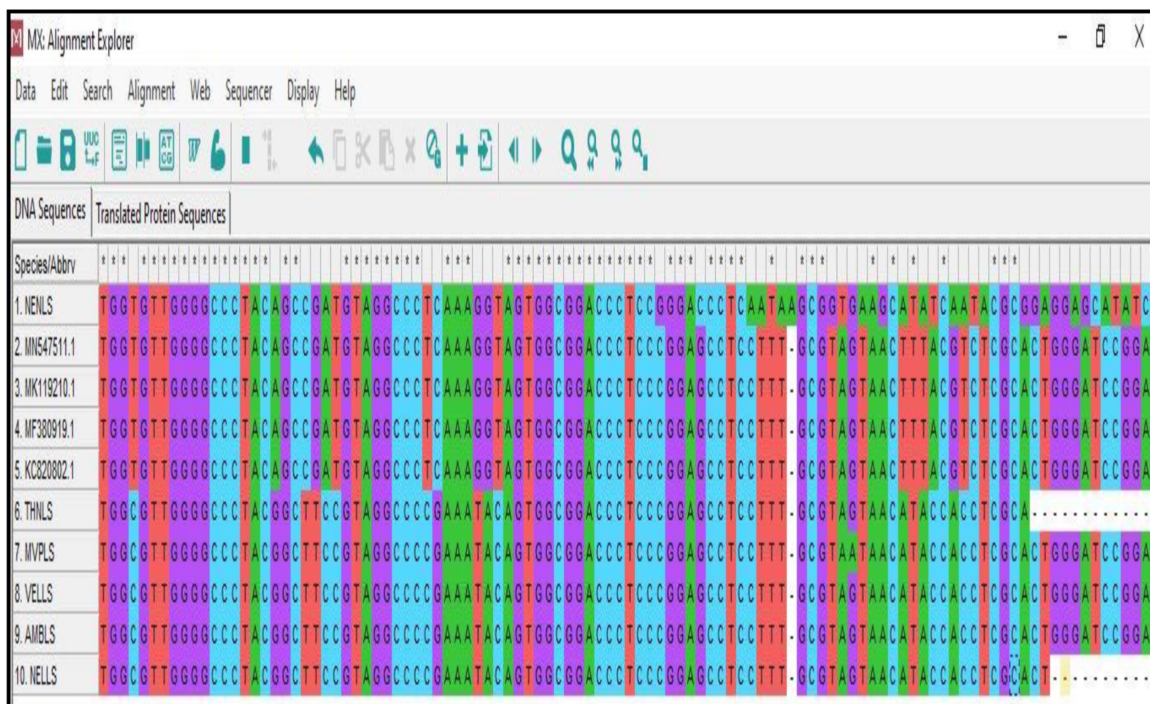


Fig 6b: Multiple Aligned sequences of *C. queenslandicum* and *C. brevisporum*

Fig. 7: Barcodes to differentiate *C. brevisporum* and *C. queenslandicum*

	Isolates	467	475	476	478	482	483	484	487	490	501	505	506	509	517	518	520	521	530	729	731	738	752	755	756	757	758	767	768	772	773	774
	NENLS	C	C	C	C	G	C	G	T	G	A	T	A	C	G	A	T	T	T	T	T	T	A	C	G	A	T	T	C	G	G	T
C. queenslandicum	**MN547511**	C	C	C	C	G	C	G	T	G	A	T	A	C	G	A	T	T	T	T	T	T	A	C	G	A	T	T	C	G	G	T
C. queenslandicum	**MK119210**	C	C	C	C	G	C	G	T	G	A	T	A	C	G	A	T	T	T	T	T	T	A	C	G	A	T	T	C	G	G	T
C. queenslandicum	**MF380919**	C	C	C	C	G	C	G	T	G	A	T	A	C	G	A	T	T	T	T	T	T	A	C	G	A	T	T	C	G	G	T
C. queenslandicum	**KC820802**	C	C	C	C	G	C	G	T	G	A	T	A	C	G	A	T	T	T	T	T	T	A	C	G	A	T	T	C	G	G	T
C. brevisporum	**THNLS**	G	T	T	T	C	G	C	C	A	C	C	C	T	A	T	G	C	C	A	C	C	G	T	T	C	C	C	G	T	A	C
C. brevisporum	**MVPLS**	G	T	T	T	C	G	C	C	A	C	C	C	T	A	T	G	C	C	A	C	C	G	T	T	C	C	C	G	T	A	C
C. brevisporum	**VELLS**	G	T	T	T	C	G	C	C	A	C	C	C	T	A	T	G	C	C	A	C	C	G	T	T	C	C	C	G	T	A	C
C. brevisporum	**AMBLS**	G	T	T	T	C	G	C	C	A	C	C	C	T	A	T	G	C	C	A	C	C	G	T	T	C	C	C	G	T	A	C
C. brevisporum	**NELLS**	G	T	T	T	C	G	C	C	A	C	C	C	T	A	T	G	C	C	A	C	C	G	T	T	C	C	C	G	T	A	C

4.6.2 Bacterial pathogen

Based on cultural, morphological and biochemical characters the bacteria associated with wilt (MNPWt and KANWt) collected from Manjapra and Kannara was identified as *Ralstonia solanacearum*. The identity of the pathogen was confirmed at the molecular level by the isolation of genomic DNA and amplification of 16 S rRNA region of the bacterial genome.

4.6.2.1 Bacterial DNA isolation

Genomic DNA of *Ralstonia solanacearum* was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel).

4.6.2.2 PCR amplification and gel electrophoresis

The amplification of 16S rRNA region of *Ralstonia solanacearum* was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). The amplification profile was 95 ºC for 5.00 min followed by 35 cycles of 95 ºC for 30 sec, 60 ºC for 40 sec and 72 ºC for 60 sec a final termination of 72 ºC for 7.00 min. The PCR products were checked in 1.2 per cent agarose gel prepared in 0.5X TBE buffer containing 0.5 µg/ml ethidium bromide and electrophoresis was performed at 75V power supply with 0.5X TBE as electrophoresis buffer for about 1-2 hours, until the bromophenol blue front had migrated to almost the bottom of the gel. The gels were visualized in a UV transilluminator (Genei) and the image was captured under UV light using Gel documentation system (Bio-Rad). The Gel image revealed that amplicon of 1.5 kB length of the 16S rRNA region was obtained (Plate 28).

4.6.2.3 In silico analysis

In silico analysis of the sequence retrieved (Fig. 8a) revealed that, the isolate showed maximum homology of 97 per cent with *R. solanacearum* (Accession no. MF973211). Thus, confirmed the bacterial wilt pathogen of passionfruit as *Ralstonia solanacearum* (Fig 8).

4.6.2.4 Phylogenetic analysis

The phylogenetic analysis revealed that the isolate from the wilt sample showed maximum relationship with the accessions KP017459, KY773618 and KX146476 and

Sequences producing significant alignments Download Manage Columns Show 100

select all 100 sequences selected GenBank Graphics Distance tree of results

Description	Max Score	Total Score	Query Cover	E value	Per. Ident	Accession
Ralstonia solanacearum strain APK76 16S ribosomal RNA gene, partial sequence	1581	1581	74%	0.0	97.42%	MF973211.1
Ralstonia solanacearum strain KAK51 16S ribosomal RNA gene, partial sequence	1450	1450	72%	0.0	95.71%	MF973210.1
Ralstonia solanacearum partial 16S rRNA gene, isolate Rs11	1443	1443	69%	0.0	96.87%	HG425355.1
Ralstonia solanacearum partial 16S rRNA gene, isolate Rs5	1203	1203	58%	0.0	96.81%	LN794229.1
Ralstonia solanacearum strain IBSBF 2571 chromosome, complete genome	1085	3238	100%	0.0	82.63%	CP026307.1
Ralstonia solanacearum strain DBCAP4 16S ribosomal RNA gene, partial sequence	1044	1044	100%	0.0	82.16%	KP017459.1
Ralstonia solanacearum strain T25 chromosome, complete genome	1038	3105	100%	0.0	82.09%	CP023014.1
Ralstonia solanacearum genome assembly 9 genomes, chromosome : II	1038	1038	100%	0.0	82.09%	LN899820.1
Ralstonia solanacearum strain B5 16S ribosomal RNA gene, partial sequence	1038	1038	100%	0.0	82.06%	KT359576.1
Ralstonia solanacearum strain B4 16S ribosomal RNA gene, partial sequence	1038	1038	100%	0.0	82.06%	KT359575.1
Ralstonia solanacearum strain RS3 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	82.04%	KM502211.1
Ralstonia solanacearum strain G12--9 16S ribosomal RNA gene, partial sequence	1035	1035	100%	0.0	82.03%	KJ494377.1
Ralstonia solanacearum strain Dong Thap 9 16S ribosomal RNA gene, partial sequence	1033	1033	100%	0.0	82.01%	MK041548.1
Ralstonia solanacearum strain P824 chromosome MP	1033	1033	100%	0.0	82.01%	CP025742.1
Ralstonia solanacearum strain P824 chromosome CM	1033	3100	100%	0.0	82.01%	CP025741.1
Ralstonia solanacearum strain SL2330 plasmid unnamed, complete sequence	1033	1033	100%	0.0	82.01%	CP022795.1

Fig 8: BLASTn text output of nucleotide sequence of 16 S rRNA region of *Ralstonia solanacearum*

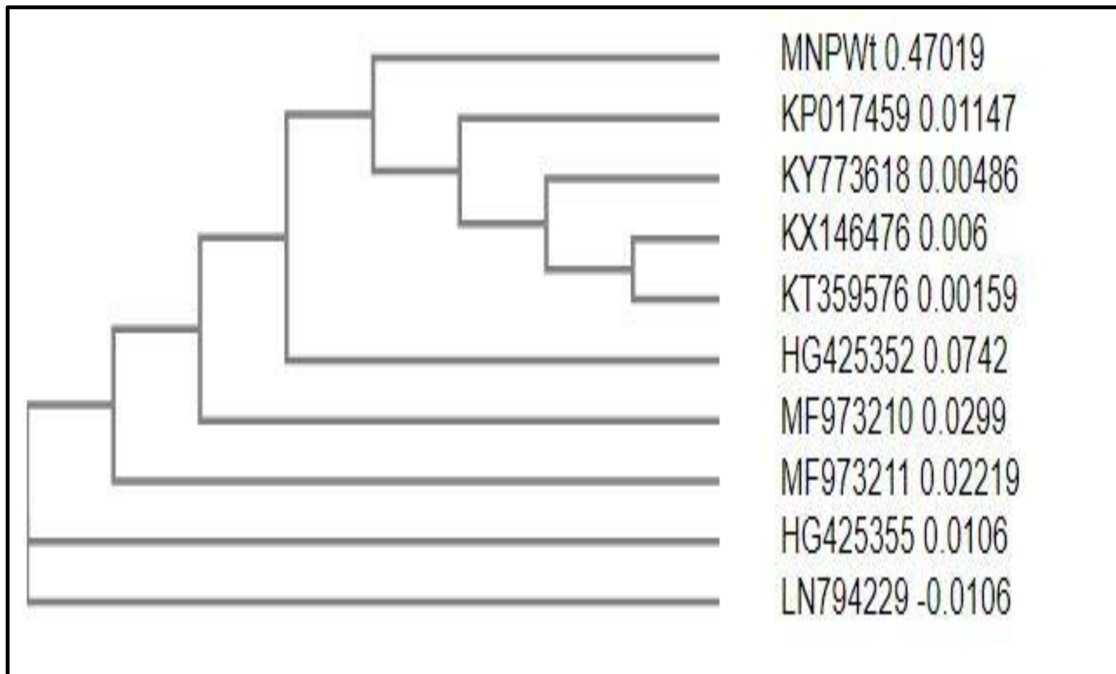


Fig 9: Phylogenetic analysis of *Ralstonia solanacearum*

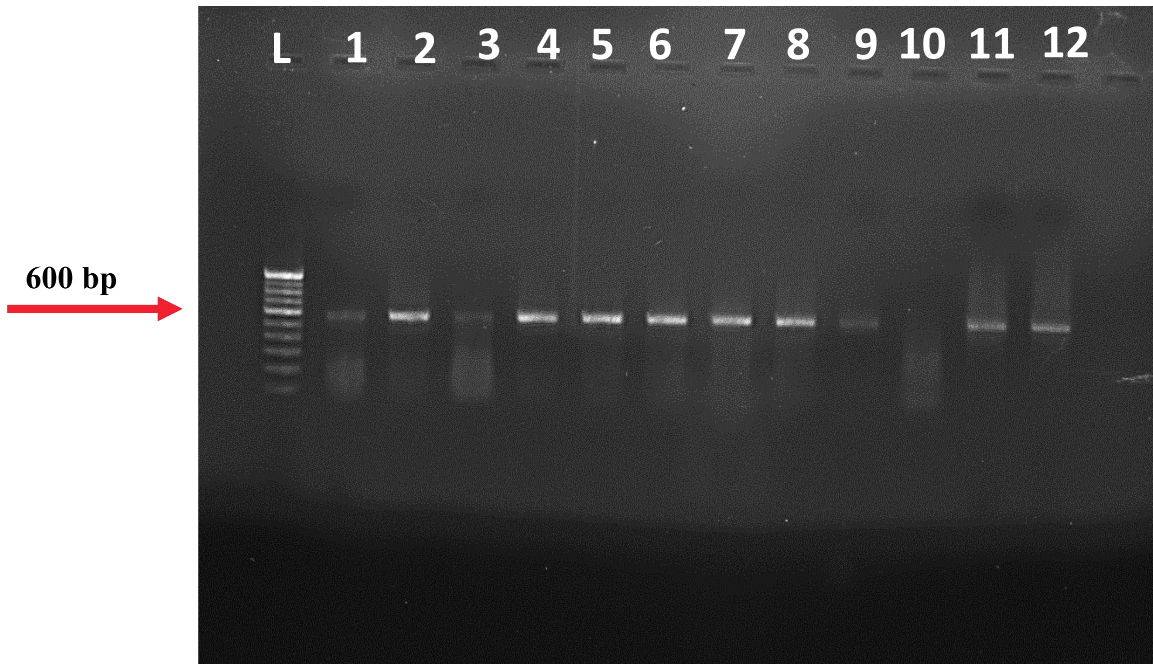


Plate 27: Standardisation of annealing temperature of *Alternaria* sp.

L- 100 BP ladder

1- 50.0° C	7 - 4.5° C
2- 50.2° C	8 - 55.5° C
3- 50.7° C	9 - 56.5° C
4- 51.5° C	10 - 57.3° C
5- 52.5° C	11- 57.8° C
6- 53.5° C	12- 58.0° C

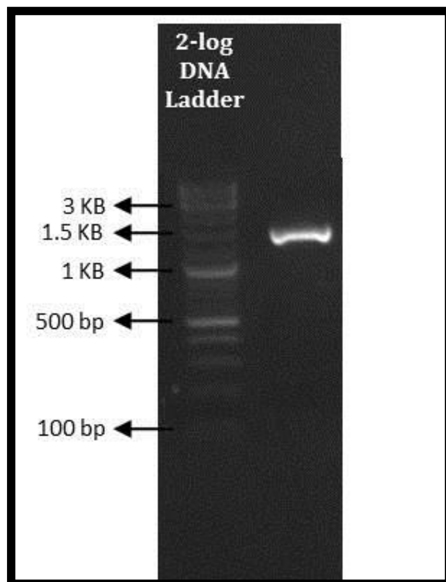


Plate 28: Gel profile of PCR-

Ralstonia solanacearum

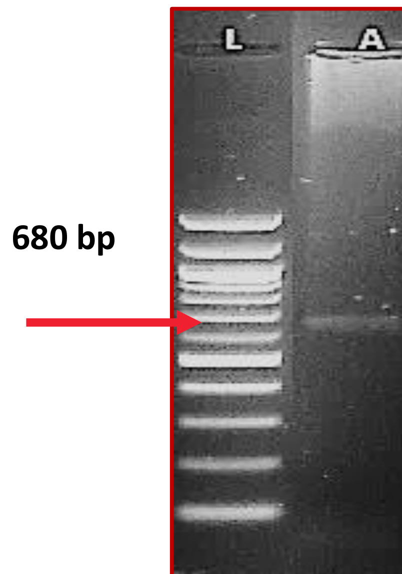


Plate 29: Gel profile of PCR-

***Potyvirus* (CI region)**

KT 359576 (Fig 9).

4.6.3 Viral pathogen

Biological and morphological characterisation revealed that the pathogen associated with leaf malformation of passion fruit as *Potyvirus*. Molecular diagnosis of the virus was done by the isolation of total RNA from the symptomatic leaf sample and amplification of region specific to the cylindrical inclusions (CI) in the viral genome.

4.6.3.1 Isolation of RNA

In order to carry out molecular detection, the isolation of total RNA was carried out from the symptomatic leaf sample and healthy leaf sample of passion fruit plants by TRIzol reagent method. The total RNA isolated was quantified using Nanodrop spectrophotometer. The concentration and the absorbance ratios of the RNA isolated were analysed and RNA sample with an absorbance value ($A_{260/280}$) greater than 1.8 was considered as good quality RNA. Gel documentation of the isolated total RNA was done using Univec Gel Documentation system and the image revealed three distinct band of rRNA without degradation.

4.6.3.2 Synthesis of first strand complementary DNA (cDNA)

The virus associated with the leaf malformation of passion fruit plants was confirmed as a positive sense RNA virus. Hence for the molecular detection through PCR, reverse transcription was performed to convert RNA to cDNA. The conversion of RNA to cDNA using Revert Aid First Strand cDNA synthesis kit was described under section 3.6.3.4. The cDNA obtained was quantified and the ratio of the absorbance value ($A_{260/280}$) was recorded to assess the purity of cDNA (Table 4.13)

Table 4.13: Quantity and quality of cDNA

Sample	Concentration (ng/µl)	Absorbance ratio ($A_{260/280}$)
1	1586.9	1.66
2	1941.6	1.57
3	1866.2	1.57

4.6.3.3 Standardisation of Reverse Transcriptase –Polymerase Chain Reaction (RT-PCR)

RT-PCR conditions such as dilution of template cDNA and annealing temperature were standardized simultaneously and the results are given below

4.6.3.3.a Standardisation of template cDNA dilution

RT-PCR was carried out at different dilutions of template cDNA such as 1:0.1, 1:0.25, 1:0.5 and 1:1 dilution. Bands were obtained only for the dilution of 1:1.

Table 4.14: Effect of dilution of cDNA on RT-PCR

Template cDNA dilutions	Nature of amplification
1:1	Amplified
1:0.5	No amplification
1:0.25	No amplification
1:0.1	No amplification

4.6.3.3.b Standardisation of annealing temperature

The annealing temperature for the RT-PCR was determined by performing the gradient PCR of 41.1, 42.7, 45.9, 48.9 and 50.2 °C and amplification was obtained at 42.7°C.

Table 4.15: Standardisation of annealing temperature for RT-PCR

Temperature	Result
41.1°C	No amplification
42.7°C	Amplified with good bands
45.9°C	No amplification
48.9°C	No amplification
50.2°C	No amplification

4.6.3.4 Detection of Potyvirus by RT-PCR

RT-PCR was carried out using reported primers (Ha *et al*., 2008) specific to

CI region of *Potyvirus* genome described under section 3.6.3.5. The reaction mixture was prepared and the composition was specified under section 3.6.3.5. The reaction with the annealing temperature 42.7°C yielded amplified products of expected band size 680bp when visualized using gel documentation (Plate 29). Hence, the virus associated with the leaf malformation of passion fruit was diagnosed and confirmed as *Potyvirus*.

4.7 CORRELATION OF WEATHER PARAMETERS WITH DISEASE SEVERITY OF FUNGAL DISEASES

Based on cultural and morphological characters, it was identified that majority of the isolates belonged to the fungal pathogens *i.e.* 14 out of 17 isolates were fungal pathogens. Therefore, the per cent disease severity of fungal diseases which were collected during the survey conducted in five districts of the state was correlated with weather parameters to identify the effect of these parameters on diseases severity. For the analysis, weather parameters like maximum and minimum temperature, relative humidity and rainfall prevailing in the surveyed location were collected from the official site of Marksim (Table 4.16) for different locations during the period of survey. Effect of each parameter on severity of fungal diseases was separately calculated using correlation analysis.

4.7.1 Effect of rainfall on severity of fungal diseases

The average value of rainfall for three months preceding the month of survey was used to correlate with the disease severity of fungal diseases. The analysis revealed that, rainfall has a strong positive correlation (Fig 10a) with the severity of fungal diseases and the correlation coefficient was 0.895**. In the case of fungal diseases, the highest PDS of 58.73 per cent and 63.90 per cent was observed for the leaf spot (MVPLS) and leaf blight (MVPLB) respectively collected from Muvattupuzha of Ernakulam district where the amount of rainfall was higher.

4.7.2 Effect of relative humidity on fungal diseases

The effect of relative humidity on the severity of fungal diseases was analyzed by correlation analysis using the average relative humidity values with the per cent disease severity. Relative humidity also showed a positive correlation (Fig 10b)

with the severity of fungal diseases with a correlation coefficient of 0.795**. Therefore, the severity of fungal disease increases with the increase in relative humidity.

4.7.3 Effect of temperature on severity of fungal diseases

Unlike rainfall and relative humidity, temperature of the location showed a negative correlation (Fig 10c) with the severity of the disease and with a correlation coefficient of (–) 0.7538**. Therefore, as temperature increased the severity of the disease decreased.

Table 4.16: Meteorological data of the surveyed locations

Sl. No.	Fungal Isolates	Locations	PDI (%)	PDS (%)	Mean Temperature (°C)	Relative humidity (%)	Rainfall (mm per day)
1.	VKALS	Vellanikkara	32.48	18.47	27.49	82.58	7.04
2.	MDKLS	Madakkathara	25.00	16.28	28.84	54.98	0.26
3.	MDKFR	Madakkathara	39.00	13.00	28.84	54.98	0.26
4.	PAZFR	Pazhayannur	53.59	26.47	25.65	89.50	11.67
5.	MNPLS	Manjapra	14.80	9.25	30.75	68.34	2.01
6.	MVPLS	Muvattupuzha	43.00	58.73	24.26	89.41	12.46
7.	MVPLB	Muvattupuzha	78.00	63.90	24.26	89.41	12.46
8.	MVPFR	Muvattupuzha	32.70	45.86	23.46	89.41	12.46
9.	NELLS	Nelliyampathy	88.75	38.53	25.85	82.45	5.94
10.	NELFR	Nelliyampathy	78.25	36.76	25.85	82.45	5.94
11.	NENLS	Nemmara	75.00	35.49	25.85	82.45	5.94
12.	NENLB	Nemmara	95.00	39.66	25.85	82.45	5.94
13.	AMBLS	Ambalawayal	48.90	40.23	23.15	88.43	9.64
14.	THNLS	Thankamani	40.84	39.92	24.04	84.70	8.67

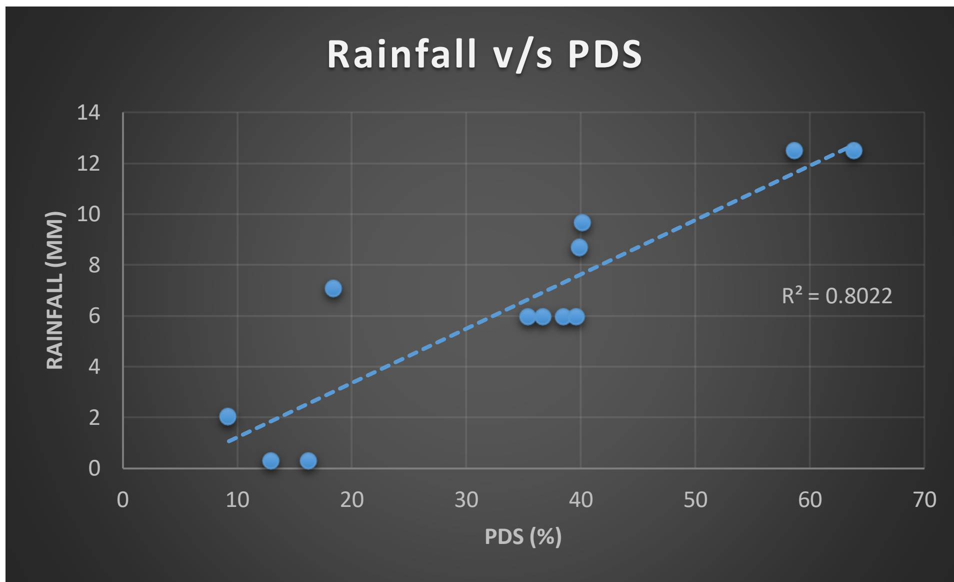


Fig 10a: Correlation of rainfall with the severity of fungal diseases

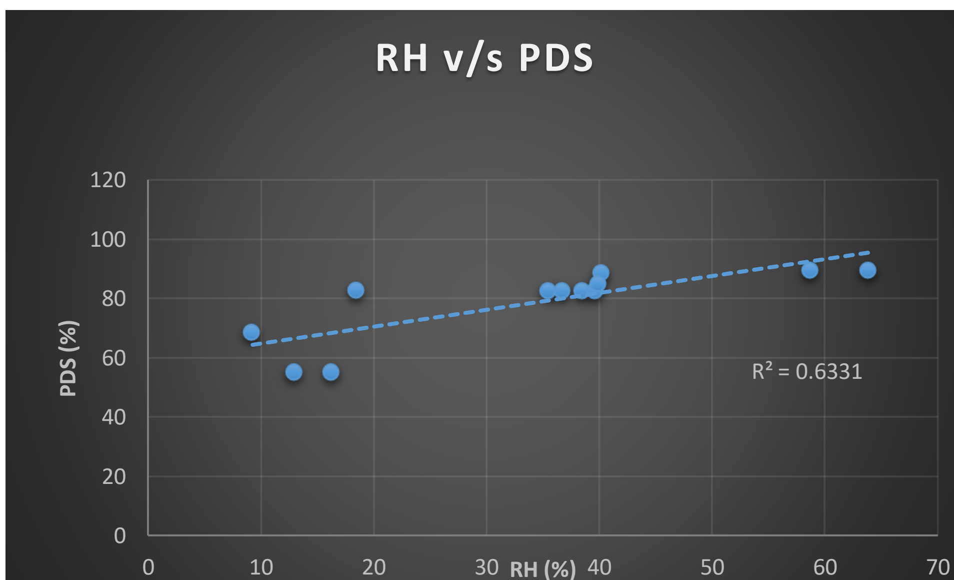


Fig 10b: Correlation of relative humidity with the severity of fungal diseases

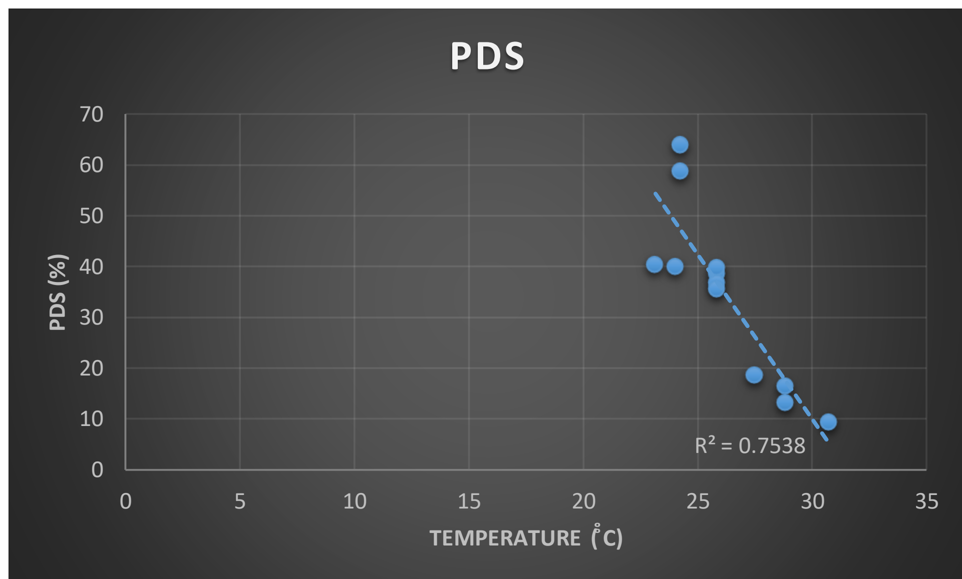


Fig 10c: Correlation of temperature with the severity of fungal diseases

4.8 *In vitro* EVALUATION OF FUNGICIDES AND BIOCONTROL AGENTS AGAINST MAJOR FUNGAL PATHOGENS

Efficacy of different fungicides and bio control agents against one foliage and one fruit rot pathogen was carried out by poison food technique. Foliage pathogen (*C. brevisporum*) and fruit rot pathogen (*S. rolfsii)* were selected as major fungal pathogens for *in vitro* studies because majority of the isolates collected were *C. brevisporum* and in the case of fruit diseases, maximum diseases severity was recorded for the fruit rot diseases caused by *S. rolfsii.*

4.8.1 *In vitro* evaluation of Fungicides

Seven fungicides at three different doses and 1 per cent Bordeaux mixture was used for poison food technique *i.e* copper hydroxide (0.05, 0.1, 0.2%), hexaconazole (0.1, 0.2, 0.3%), propineb (0.15, 0.25, 0.35%), difenoconazole (0.025, 0.05, 0.075%), carbendazim 12% + mancozeb 64% (0.1, 0.2, 0.3%), cymoxanil8% + mancozeb 64% (0.1, 0.25, 0.5%), azoxystrobin (0.005, 0.01, 0.015%) and Bordeaux mixture (1.0%) against *C. brevisporum* and *S. rolfsii.*

4.8.1.1 In vitro evaluation of fungicides against S. rolfsii (Plate 30)

Two fungicides *i.e.* azoxystrobin (0.005, 0.01, 0.015%) and hexaconazole (0.1, 0.2, 0.3%) were cent per cent effective against *S. rolfsii* at lower, recommended and higher doses. The combination of cymoxanil and mancozeb (Curzate) showed 100 per cent inhibition at higher dose *i.e.* 0.5 per cent whereas at recommended dose (0.25%), the fungicide showed 80.29 per cent inhibition and at lower dose of 0.1 per cent, 64.17 per cent inhibition was noticed. Difenaconazole when applied at lower dose (0.025%) resulted in 85.28 per cent inhibition with a colony diameter of 1.325 cm. At recommended dose of 0.05 per cent, an inhibition of 88.89 per cent was observed and at higher dose of 0.075 per cent, cent per cent inhibition was observed. An inhibition per cent of 75.28, 87.50 and 100 was observed when propineb was used at lower (0.15%), recommended (0.25%) and higher doses (0.35%) respectively. Copper hydroxide resulted in 85.83, 88.89 and 100 per cent inhibition when applied at three doses *i.e.* lower (0.05%), recommended (0.1%) and higher doses of 0.2 per cent respectively. The combination of carbendazim and mancozeb recorded only 43.66 per

cent and 77.44 per cent inhibition when applied at lower (0.1%) and recommended dose (0.2%). But at a higher dose of 0.3 per cent, it gave cent per cent inhibition of the pathogen. Bordeaux mixture (1%) was not that effective against *S. rolfsii*, it only showed an inhibition of less than 50 per cent *i.e.* 47.5 per cent.

4.8.1.2 In vitro evaluation of fungicides against C. brevisporum (Plate 31)

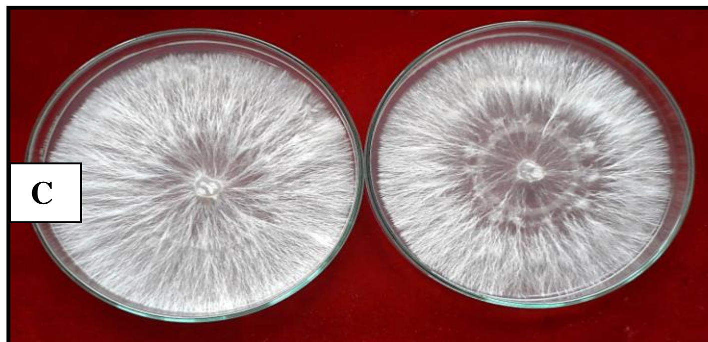
Copper hydroxide, hexaconazole and a combination of carbendazim and mancozeb were highly effective against *C. brevisporum* at all the three doses *i.e.* higher, recommended and lower doses. In all the cases cent per cent inhibition was noticed. Propineb and azoxystrobin showed cent per cent inhibition at recommended and higher doses. At lower dose of 0.015 per cent (propineb) and 0.005 per cent (azoxystrobin), 77.11 per cent and 86.66 per cent inhibition was recorded. The combination of cymoxanil and mancozeb resulted in 23.56 per cent, 41.67 per cent and 73.16 per cent inhibition at lower (0.1%), recommended (0.25%) and higher (0.5%) doses respectively. Difenaconazole when applied at lower (0.025%), recommended (0.05%) and higher (0.1%) doses recorded 87.11 per cent, 88.33 per cent and 89.08 per cent respectively.

4.8.2 *In vitro* evaluation of Bio control agents

Biocontrol agents *viz. Trichoderma* sp. (KAU reference culture) and *Pseudomonas fluorescence* (KAU reference culture) were tested *in vitro* by dual culture technique (Johnson and Curl, 1972) for the control of *S. rolfsii* (Fruit rot pathogen) and *C. brevisporum* (Leaf spot pathogen) of passion fruit collected during the survey (Plate 32).

It was evident from the experiment that *Trichoderma* sp. was capable of inhibiting the growth of *S. rolfsii* and *C. brevisporum.* An inhibition of 50 per cent was observed for *S. rolfsii* by the antagonist *Trichoderma* sp. When the same was used against *C. brevisporum,* cent per cent inhibition was observed.

P. fluorescens, the bacterial antagonist produced an inhibition of 72.22 per cent when used against *C. brevisporum*. Inhibition of the growth of *S. rolfsii* was not observed when *P. fluorescens* was used.



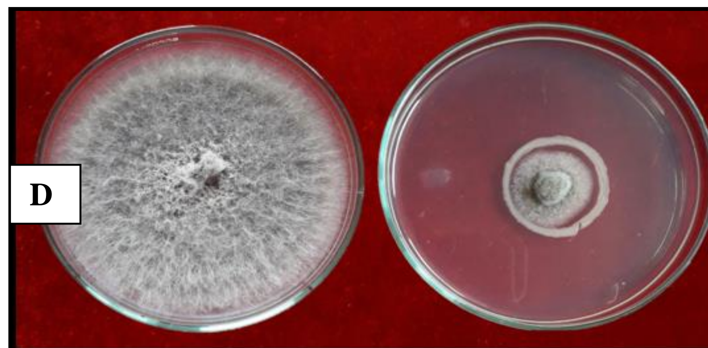


Plate 32: ***In vitro*** **evaluation of biocontrol agents**

- **A.** *Trichoderma* sp. X *S. rolfsii*
- **B.** *Trichoderma* sp. X *C. brevisporum*
- **C.** *P. fluorescens* X *S. rolfsii*
- **D.** *P. fluorescens* X *C. brevisporum*

Table 4.17: Per cent Inhibition of major fungal pathogens by Biocontrol agents

Sl. No.	**Biocontrol agent / Pathogen**	*Trichoderma* sp.	*Pseuodomonas fluorescens*
1.	*Sclerotium rolfsii*	50%	0%
2.	*Colletotrichum brevisporum*	100%	72.22%

Table 4.18: *In vitro* evaluation of fungicides against *Sclerotium rolfsii*

Sl. No.	Fungicide	Concentration (%)	Mean colony diameter (cm)	Inhibition (%)	Transformed value *
1.	Copper hydroxide	0.05	1.28	85.83	67.893^{bc}
		0.10	1.0	88.89	70.528^{b}
		0.20	0	100.00	87.134^{a}
2.	Hexaconazole	0.10	0	100.00	87.134^{a}
		0.20	0	100.00	87.134^{a}
		0.30	0	100.00	87.134^{a}
3.	Probineb	0.15	2.23	75.28	60.181^{e}
		0.25	1.13	87.50	69.314^{bc}
		0.35	0	100.00	87.134^{a}
4.	Difenaconazole	0.025	1.33	85.28	67.534^{c}
		0.050	1.00	88.89	70.528^{b}
		0.075	0	100.00	87.134^{a}
5.	Carbendazim + Mancozeb	0.10	5.07	43.66	41.359^{g}
		0.20	2.03	77.44	61.989^{de}
		0.30	0	100.00	87.134^{a}
6.	Cymoxanil + Mancozeb	0.10	3.23	64.17	53.262^{f}
		0.25	1.78	80.28	63.638^{d}
		0.50	0	100.00	87.134^{a}
7.	Azoxystrobin	0.005	0	100.00	87.134^{a}
		0.010	0	100.00	87.134^{a}
		0.015	0	100.00	87.134^{a}
8.	Bordeaux Mixture	1.00	4.73	47.50	43.554^{g}
	CV				2.217
	CD (0.05)				2.699
	CD (0.01)				3.606**

*Angular transformed values

**Significant at 1 per cent level.

Table 4.19: *In vitro* evaluation of fungicides against *Colletotrichum brevisporum*

Sl. No.	Fungicide	Concentration (%)	Mean colony diameter (cm)	Inhibition (%)	Transformed value*
1.	Cymoxanil + Mancozeb	0.10	6.88	23.56	28.825[c]
		0.25	5.25	41.67	40.154[c]
		0.50	2.42	73.16	58.830[b]
2.	Difenaconazole	0.025	1.16	87.11	68.895[b]
		0.050	1.05	88.33	70.025[ab]
		0.075	0.98	89.08	70.699[ab]
3.	Probineb	0.15	2.06	77.11	62.151[b]
		0.25	0	100.00	87.134[a]
		0.35	0	100.00	87.134[a]
4.	Copper hydroxide	0.05	0	100.00	87.134[a]
		0.10	0	100.00	87.134[a]
		0.20	0	100.00	87.134[a]
5.	Carbendazim + Mancozeb	0.10	0	100.00	87.134[a]
		0.20	0	100.00	87.134[a]
		0.30	0	100.00	87.134[a]
6.	Azoxystrobin	0.005	1.20	86.66	68.583[b]
		0.010	0	100.00	87.134[a]
		0.015	0	100.00	87.134[a]
7.	Hexaconazole	0.10	0	100.00	87.134[a]
		0.20	0	100.00	87.134[a]
		0.30	0	100.00	87.134[a]
8.	Bordeaux Mixture	1.00	0	100.00	87.134[a]
	CV				14.161
	CD (0.05)				17.282
	CD (0.01)				23.089**

*Angular transformed value

**Significant at one per cent level of significance.

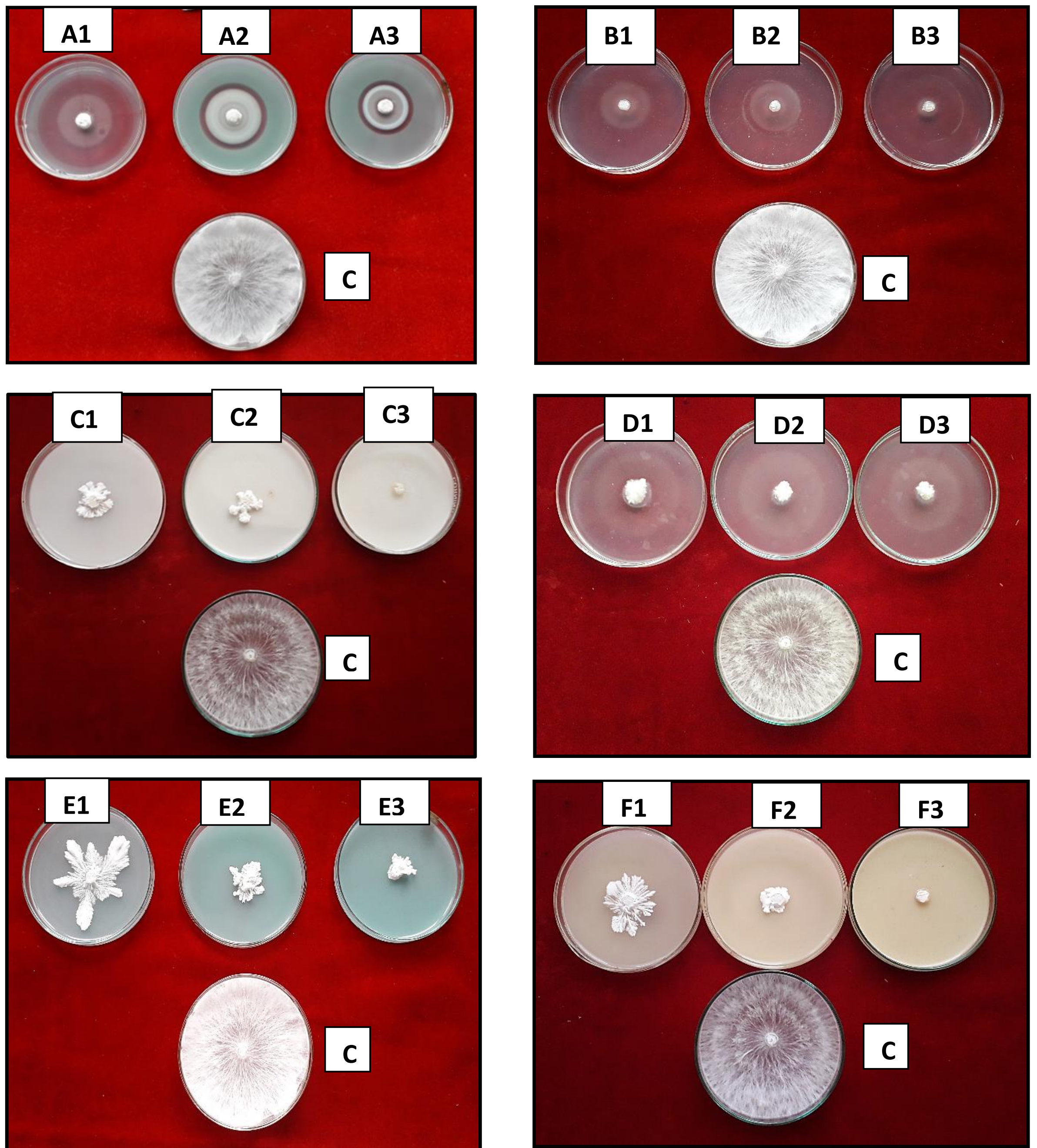


Plate 30: ***In vitro* evaluation of fungicides against *Sclerotium rolfsii***

A1: Copper hydroxide (0.05%), A2: Copper hydroxide (0.1%), A3: Copper hydroxide (0.2%)

B1: Hexaconazole (0.1%), B2: Hexaconazole (0.2%), B3: Hexaconazole (0.3%)

C1: Propineb (0.15%), Propineb (0.25%), Propineb (0.35%)

D1: Difenoconazole (0.025%), Difenoconazole (0.05%), Difenoconazole (0.075%)

E1: Carbendazim 12% + mancozeb 64% (0.1%), E2: Carbendazim 12% + mancozeb 64% (0.2%), E3: Carbendazim 12% + mancozeb 64% (0.3%)

F1: Cymoxanil 8% + mancozeb 64% (0.1%), F2: 0.5Cymoxanil 8% + mancozeb 64% (0.25%), F3: Cymoxanil 8% + mancozeb 64% (0.5%) C: Control plate

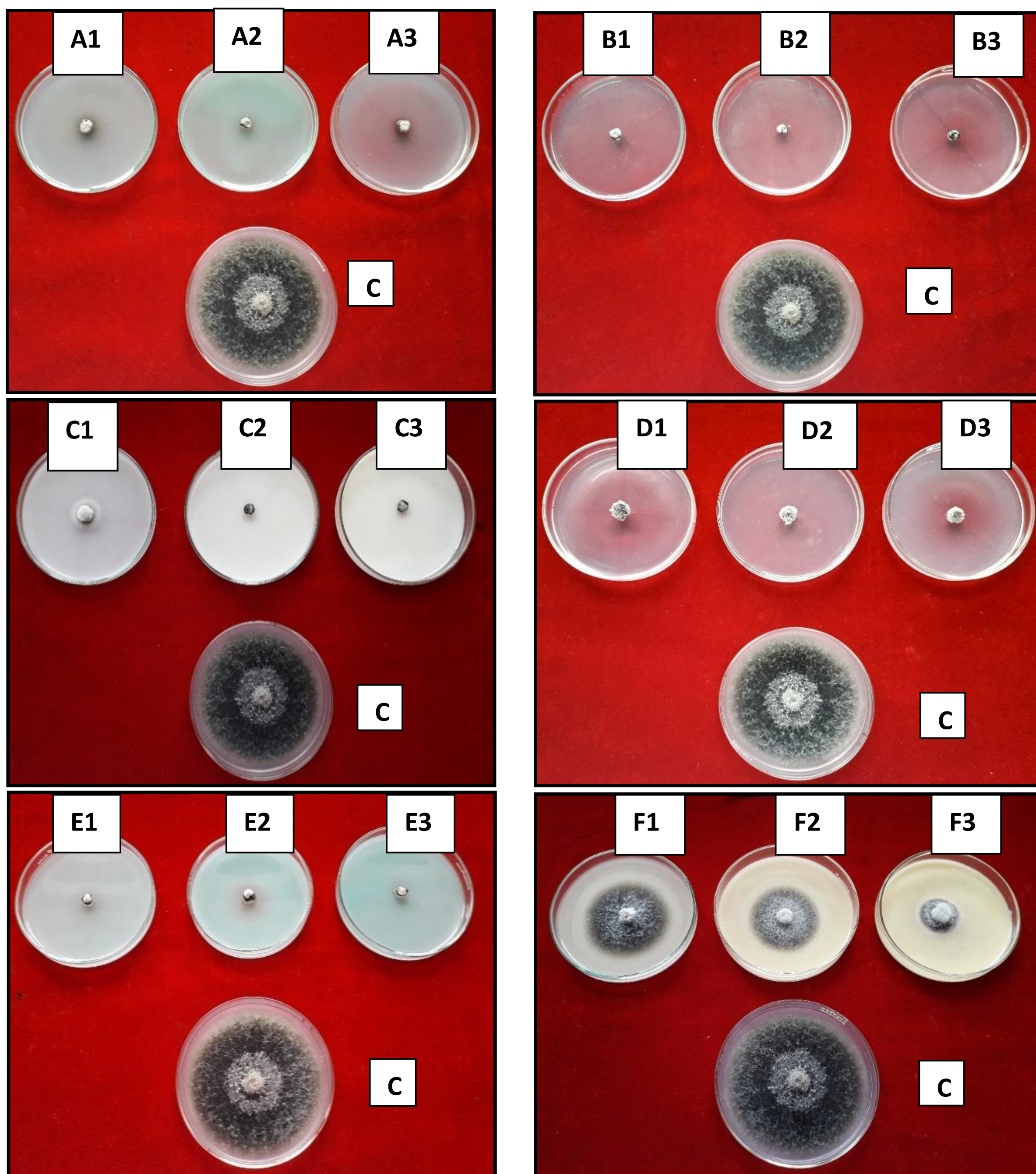


Plate 31: ***In vitro* evaluation of fungicides against *Colletotrichum brevisporum***

A1: Copper hydroxide (0.05%), A2: Copper hydroxide (0.1%), A3: Copper hydroxide (0.2%)

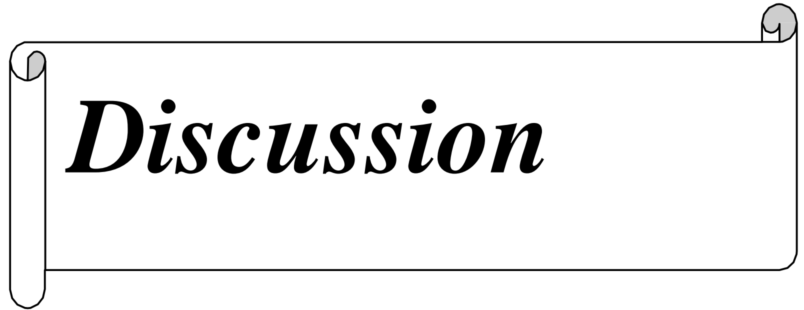
B1: Hexaconazole (0.1%), B2: Hexaconazole (0.2%), B3: Hexaconazole (0.3%)

C1: Propineb (0.15%), Propineb (0.25%), Propineb (0.35%)

D1: Difenoconazole (0.025%), Difenoconazole (0.05%), Difenoconazole (0.075%)

E1: Carbendazim 12% + mancozeb 64% (0.1%), E2: Carbendazim 12% + mancozeb 64% (0.2%), E3: Carbendazim 12% + mancozeb 64% (0.3%)

F1: Cymoxanil 8% + mancozeb 64% (0.1%), F2: 0.5Cymoxanil 8% + mancozeb 64% (0.25%), F3: Cymoxanil 8% + mancozeb 64% (0.5%)   C: Control plate


Discussion

5. DISCUSSION

Fruits are widely accepted and considered as an important component of healthy diet and its adequate consumption could help to reduce a wide range of diseases (Craig and Beck, 1999). Boosting the fruit production to increase availability, affordability, access and consumption of fruits for maximum health benefits is of global concern. Passion fruit (*Passiflora edulis* Sims.) is an attractive option for health conscious people and have nutritious fruit which are highly appreciated for fresh consumption and industrial purposes because of its diverse uses for juice, jelly and ice cream products (Santos, 2002). The fruit has high nutritional and medicinal value. It is a rich source of Vitamin A, C and contain fair amounts of iron, potassium, sodium, magnesium, sulphur, chlorides, dietary fibre and protein. The fruits which are nearly round in shape with a smooth, waxy, tough rind and weighing about 35 to 40g in yellow varieties and about 60g in purple varieties. The edible portion is an aromatic mass of double-walled, membranous sacs containing orange coloured pulpy juice with about 250 small, dark brown to black pitted seeds inside the fruit (Thokchom and Mandal, 2017). India enjoys harvest of passion fruit in Nilgiris, Wayanad, Kodaikanal, Shevroys, Coorg and Malabar of south and Himachal of northern India and North Eastern states like Manipur, Nagaland Mizoram and Meghalaya. Considering the interest of people and the suitability of agro climatic conditions of the state, passion fruit is a better option for the farmers of Kerala. Even though, it is a crop of great potential, the production is often constrained by many biotic challenges such as susceptibility to pests and diseases. Collar/root rot, anthracnose, brown spot and woodiness virus were the major diseases reported globally. But, a systematic study on the diseases of passion fruit was not conducted so far in the state of Kerala.

Recently, passion fruit is widely cultivated and emerged as a novel favourite fruit crop of the state, its biotic challenges need to be addressed and documented. Therefore, this project was undertaken in order to benefit the farmers for getting maximum returns from the crop by minimising the loss by effective management of diseases. For this, the preliminary step is the identification and characterisation of the pathogen associated with various disease of passion fruit grown in Kerala. Hence, the

project was initiated with purposive sampling surveys in the passion fruit growing tracts of the state in order to identify the diseases and characterize the pathogen associated.

5.1 SURVEY

Purposive sampling surveys were conducted in passion fruit growing tracts of five districts of Kerala *i.e.* Thrissur, Palakkad, Ernakulam, Wayanad and Idukki during the period of 2018 – 20 to catalogue the diseases prevailing in the crop and to characterize the associated pathogens. During these surveys, the incidence and severity of each type of symptoms observed were recorded from all the locations. Each type of symptom was catalogued by assigning a code and samples were taken for further investigation to identify and confirm the actual pathogen associated with the disease. Five different type of samples (Leaf spot, leaf blight, fruit rot, wilt, leaf malformation) were observed during the survey. Based on the symptoms observed on different plant parts, the samples from each location were catalogued and abbreviated as leaf spots (LS), leaf blights (LB), wilt (Wt), fruit rots (FR), bud blight (BB), leaf malformation (LM) *etc.* prefixed by the abbreviations assigned for each location. Maximum number of symptomatic samples were collected from Thrissur district (7 no.s) followed by Ernakulam (5 no.s), Palakkad (4 no.s), Idukki (1 no.) and Wayanad (1 no.). Minimium number of symptomatic samples were recorded from the high altitude regions. This may be because the climatic conditions prevailing in those areas were unfavourable for the disease development unlike plains.

Isolates of leaf spot (8 no.s), leaf blight (2 no.s), fruit rot (4 no.s), bud blight (1 no.) and wilt (2 no.s) and were collected from different surveyed locations. Leaf malformation symptoms similar to viral infections like puckering, cupping, vein clearing, reduction in leaf lamina and leathery appearance were found predominant in the field of Vellanikkara. Leaf spot was the common symptom prevalent in all the districts. The occurrence of similar types of leaf spot were described by Smith (1939), Ram *et al.* (1977), Yamashiro (1991), Cedeno *et al.* (1993), Lutchmeah (1993), Wolcan and Larran (2000), Manicom *et al.* (2003) and Du *et al.* (2017).

Samples of the symptomatic plant parts were labelled with the specific code and were brought to the laboratory for further investigation and based on preliminary

microscopic and cultural observations, these were identified up to the genus level by comparing the morphological and cultural characters identified with the CMI Descriptions of Pathogenic Fungi and Bacteria (CMI, 1964). Thus, the preliminary examinations revealed that leaf spot, leaf blight, bud blight and fruit rot symptoms were due to fungi and the wilting is due to pathogenic bacteria. In the case of viruses, preliminary identity was made based on diagnostic symptoms.

The incidence and severity of each type of symptoms were assessed separately from each location using the formula given by Wheeler (1969). Leaf spot was the predominant symptom in majority of the locations. VKALS, MDKLS, MNPLS, NELLS, NENLS, MVPLS, AMBLS and THNLS were the leaf spot isolates collected from the locations Vellanikkara, Madakkathara, Manjapra, Nelliyampathy, Nenmara, Muvattupuzha, Ambalavayal and Thankamani respectively. The predominance of leaf spot symptom may be due to the easiness in dissemination of spores of pathogen *via* wind, rain, insects *etc.* Out of these, MVPLS collected from Muvattupuzha recorded highest severity of 58.73 per cent with an incidence of 43 per cent. High humidity and rainfall of this location was greatly conducive for the spread of disease. VKALS, MDKLS, MNPLS, NELLS, NENLS, AMBLS and THNLS were found to occur in the fields with 18.47, 16.28, 9.25, 38.53, 35.49, 40.23, 40.84 per cent respectively.

Four fruit rot diseases MDKFR, NELFR, MVPFR and PAZFR were obtained during the purposive sampling surveys. Among these PAZFR recorded the highest severity per cent *i.e.* 26.47 with an incidence of 53.59 per cent. Fruit rot from Nelliyampathy (NELFR) showed highest incidence of 78.25 but the severity is 24.76 per cent only. The fruit rot observed in Muvattupuzha showed a severity of 20.39 with an incidence of 45.86. MDKFR was recorded an incidence of 39 per cent but the severity was only 13 per cent.

From the preliminary microscopic observations, VKALS 1, NELLS, NENLS, MVPLS, AMBLS, THNLS, NELFR and MVPFR were found to be caused by fungal pathogen, *Colletotrichum* sp. Therefore, this can be considered as the most important disease which occurs in all the passion fruit growing tracts of Kerala. Yamashiro (1991), Cedeno *et al.* (1993), Lutchmeah (1993), Wolcan and Larran (2000) also

described anthracnose caused by *Colletotrichum* as one of the major disease of passion fruit which occurred wherever the crop was grown.

Wilting of the entire vines was observed in locations like Angamaly and Kannara where the incidence was 37.64 and 33 per cent respectively. The disease has predominant status because the infected plants were completely lost due to the disease and thereby causing huge economic loss to the farmers.

A fruit and leaf malformation in passion fruit was observed in one of the surveyed fields in Vellanikkara. The incidence and severity of the symptom was 89.94 and 75.38 per cent respectively. The highest severity of the leaf malformation had a negative influence on the yield and harvest from the infected vines as the infection resulted in complete loss of vigour of the plant.

5.2 PATHOGENICITY

All the fungal and bacterial pathogenic isolates collected from the surveyed locations were subjected for pathogenicity tests by establishing Koch's postulates. Different types of techniques were employed for the inoculation of isolates based on type of pathogen (fungi or bacteria) and nature of symptoms. Mycelial Bit Inoculation Method (MBIM) was used for proving pathogenicity of foliage and fruit diseases in which mycelial plugs of fresh cultures of different isolates of the pathogen were placed over the surface of leaf as given by Rocha *et al.* (1998).

The leaf spot pathogen isolates from VKALS, NELLS, NENLS, MVPLS, AMBLS, THNLS were found to infect and produce symptoms the leaf tissues within 2- 3 days when artificially inoculated which was similar to the result obtained by Chen and Huang (2018) where the typical anthracnose symptoms were initiated after three days of inoculation with the mycelial plugs.

The other leaf spot isolates from leaf spot samples MDKLS and MNPLS were also employed the same MBIM and the symptoms were started to appear within 4 days and 2 days respectively. The typical symptoms of black spots with concentric zonations and black lesions were seen on the inoculated leaves for the isolate from MDKLS and MNPLS within one week of inoculation and these observation was in congruence with the results of Guevara *et al.* (2019) and Vico *et al.* (2017) respectively. MVPLB and

NENLB were leaf blight samples and these isolates when inoculated on detached leaves initiated the infection within 4 days and 3 days respectively (Rahayu, 2014).

Four isolates from MDKFR, NELFR, PAZFR and MVPFR collected during the survey were subjected to the pathogenicity establishment studies following the same protocol of MBIM and the same protocol was also replicated by Firmino *et al*. (2013). MDKFR isolate initiated the infection within 4 days of inoculation and produced the concentric zonation after 12th day. The fruit rot isolate from Nelliyampathy, NELFR started the infection on the inoculated fruits within 3 days of inoculation and the symptoms appeared as small brown spots initially which enlarged to water soaked large lesions finally leading to the entire rotting of the fruits. MVPFR initiated the infection within 3 days of inoculation and the symptoms initially appeared as brown spots which later enlarged and become sunken and dark brown. On progression of disease, the lesion spread to the entire fruit and the skin become papery and in the advanced stage, acervuli were formed on the fruit surface. Similar observation was recorded by Joy and Sherin (2012). PAZFR initiated the infection within 2 days of inoculation as water soaked pink coloured spots which later enlarged with white cottony mycelial mats covering the fruits after the four days of inoculation and sclerotial bodies appeared on the inoculated fruits after one week of inoculation. This observation was in congruence with the observations of Kwon *et al*. (2009).

Two isolates of bacteria associated with wilt disease of passion fruit (MNPWt and KANWt) were collected. All the isolates were proven for pathogenicity studies by root clipping followed by drenching with bacterial suspension and the symptoms started to appear after four days of inoculation. The initial symptoms were drooping of leaves followed by complete wilting and the inoculated plants died after 10 days. The symptoms expression was in line with the results obtained by Aslam *et al*. (2017) for *Ralstonia solanacearum* infecting tomato.

5.3 STMPTOMATOLOGY

Symptoms produced by all the isolates collected during the survey conducted in passion fruit growing tracts of five districts of the state were thoroughly observed and

documented both under natural field condition and on artificial inoculation (discussed in section 5.2).

Under natural conditions, VKALS 1, NELLS, NENLS, MVPLS, AMBLS and THNLS were characterized by light coloured spots at the initial stages which later turned to brown coloured large spots later coalesced to large necrotic patches. These observations were in line with those of Yamashiro (1991). In addition to these symptoms all these samples had a yellow halo around the spots and acervili were developed at the centre as the spots matured. However, MVPLS and NELLS produced brown coloured irregular spots which later enlarged to large necrotic areas with fruiting bodies produced at the centre and the isolate MVPLS was mainly concentrated to the marginal areas.

The leaf spot (MDKLS) was characterized by small minute brown spots of 0.3 - 0.5 cm size and these spots are seen throughout the leaf lamina. Similar type of symptoms was recorded by Li *et al.* (2019) in coleus infected by *Alternaria alternata*. The leaf spot (MNPLS) collected from Manjapra of Angamaly produced irregular shaped spots which were white in colour with papery texture and later the infected areas fall off giving shot hole appearance and fructifications of the fruiting bodies were also seen on spots in the advanced stages.

MVPLB and NENLB were the two leaf blight symptoms obtained through the survey. MVPLB, the isolate produced cream coloured small spots initially which later enlarged and gave rise to irregular blighted appearance and were uniformly distributed throughout the leaf lamina. In the case of NENLB, it produced extensive blighted areas in the interveinal areas and the blighted areas appear to be light coloured with papery texture having a brittle nature. Both the observations were in congruence with the symptom produced by *Rhizoctonia solani* described by Potronieri *et al.* (1999).

Rotting was mainly observed as the disease affecting fruits during the survey. Four isolates of the rot pathogen were collected *i.e.* MDKFR, NELFR, MVPFR, PAZFR. The isolate MDKFR produced light colored spots initially which later turning to brown spots and the spots remain dry rather than water soaked. The appearance of spot was found to be similar to the observation by Fullerton (1982) where he observed

that water soaked sunken spots unlike the dry spots by *Alernaria passiflorae*. The pathogen was not found to affect the pulp of the fruits but the consumer preference was found to be reduced. NELFR and MVPFR were the other two symptoms suspected to be caused by *Colletotrichum* sp. based on preliminary microscopic observation of spores from the infected samples. NELFR produced dark brown coloured spots on the mature fruits with soft and sunken rotten areas, later these lesions extended on to the large areas and affected the quality of pulp inside the fruits and fruiting bodies were seen at the centre of spots. Similar type of symptomatology caused by *Colletotrichum gloeosporoides* in passion fruit was explained by Fischer and Rezende (2008). In addition to these symptoms separation of fruit skin from the surface of fruit and along with a papery appearance was also observed in NELFR which is similar to those symptoms given by Joy and Sherin (2012). The symptoms shown by MVPFR were in congruence with those above but the colour of spot was creamy white and no separation and papery appearance of fruit skin at infected area. PAZFR produced necrosis on the fruits and in these type of rotting the initial symptom appeared as light pink coloured rotten areas with white cottony mycelial growth which later extended to produce extensive rotting of the fruits affecting the fruit pulp. Smooth, round and dark brown coloured sclerotial bodies were formed on the advanced stages of the diseases. These observations were in line with those given by Kwon *et al.* (2009) in melon fruit rot caused by *Sclerotium rolfsii*.

Two isolates of wilt causing pathogen were obtained from the sampling surveys conducted in Thrissur district. The initial symptoms include sudden wilting of the entire vines of passionfruit and the plants of all stages were infected but more incidence was observed in fruiting plants. This highlights the importance of the disease which leads to complete yield and crop loss to the farmers. The most prominent symptom is the rapid wilting of the entire vines while the leaves retained its green colour and within few days of infection, the entire plant become wilted and dried. The infection spreads to nearby vines at a rapid rate and the other symptoms found associated with the diseases are discolouration of vascular bundles and extensive rotting of roots. Ooze test was conducted to check whether the disease was fungal or bacterial. The milky white ooze containing bacterial cells from the stem cuttings of passion fruit confirmed that

the disease was due to bacteria. Chakravarty and Kalita (2011) also observed similar type of symptom from the plant infected with bacterial pathogen.

Severe symptoms like viral infections *i.e.* islands of light green and dark green areas, vein clearing, extreme reduction of leaf lamina, leathery appearance of leaf, puckering and cupping of leaf, malformed fruits, chlorotic spots and enations were found in one of the surveyed field. Similar type of symptoms was described from Australia (McKnight, 1953), Nigeria (Martini, 1962), Taiwan (Chang, 1992) and Japan (Iwai *et al*.,1996) while dealing with the passion fruit woodiness virus infecting passion fruit.

5.4 CHARACTERISATION

The pathogens associated with passion fruit were isolated and characterized by cultural and morphological characters and biochemical (in the case of bacteria) characteristics. Viral samples collected were subjected to biological characterization *i.e.* sap transmission and mechanical transmission in order to characterize the pathogen.

The fungal pathogen isolates collected from the survey were isolated on PDA medium and observed for the cultural characteristics of fungi like colour, texture, growth rate, pattern of growth, sporulations, pigmentation, colour on the back side of petri plates and presence of fruiting bodies. Slide culture technique for each isolates was carried out for various microscopic observations like colour of hyphae, branching pattern, sepatation, presence of conidia, conidial septation, types, shape and size of spores, length and breadth ratio of spores and presence of sexual structures.

Eight leaf spot isolates were collected from the five surveyed districts. Out of which six isolates i.e. VKALS, NELLS, NENLS, MVPLS, AMBLS and THNLS were identified to be caused by *Colletotrichum* sp. VKALS produced fluffy mycelial growth characterized by light grey aerial mycelium gradually turned to dark grey colour and dark grey colour was developed at the reverse side of Petri plate. As the culture matures, orange coloured spore mass was developed and when viewed under microscope, the septate hyphae appeared hyaline and the conidia were single celled, hyaline, cylindrical, bullet shaped with round and obtuse ends with a size of 8.64 µm x 3.03 µm. The cultural characteristics of VKALS were in consistent with those given by Jeffries *et al*. (1990).

Likewise, the remaining five isolates *viz* NELLS, NENLS, MVPLS, AMBLS and THNLS also produced fluffy dull white to light grey coloured mycelium which later turned to dark grey coloured and the reverse side of the petri plate showed dark grey to black colour. All the isolates produced hyaline mycelium with septations. The conidia produced were bullet shaped with round and obtuse ends and oil globules can be seen at the centre of conidia. The size of conidia ranged from 7.38 - 10.67 µm x 2.67-3.57 µm. Similar conidial characteristics were described by Wolcan and Larran (2000) and Peres *et al.* (2002) confirming the pathogen as *Colletotrichum* sp. All the isolates completed the growth within 7-8 days in the Petri plate when incubated at room temperature and the growth rate was found to be 1. 13 to 1.29 cm per day.

MDKLS, another leaf spot pathogen from Madakkathara was confirmed to be *Alternaria* sp. which was characterized by the formation of sub surface mycelium with whitish grey colour and the pathogen completed the growth in the petri plate by 11-12 days. Alternate zones were visible at the reverse side of the petri plate and appeared black in colour. The cultural characteristics of *Alternaria* sp. on PDA medium were in line with the findings of Kapoor and Hingorani (1958) and Nagrale *et al*. (2013). Brown coloured septate mycelia were produced by the pathogen with abundant conidia production and the conidia were brown in colour with transverse and longitudinal septa (muriform) with an average size of 33.28 µm length and 13.63 µm width and a prominent beak of 1.04 µm length. Conidial characeristics observed in this study were found similarity with the observations recorded by Ploetz *et al.* (2003).

MNPLS caused by *Diplodia* sp. developed white to smoky grey colonies on PDA within 3 days when incubated at room temperature and mycelium showed fluffy aerial growth which later turns to greyish black followed by deep black colour. When the culture matures, tough black coloured tarry structures were developed inside the petri plates. The hyphae appeared brown to black coloured with the production of single celled conidia at the initial stage and were ellipsoidal or obovate with thick walls. The colour of conidia was brown and these conidia developed single septa which divides the conidia into two with 28.32 µm length and 14.19 µm width as conidial dimensions. In this study, the size of conidia was nearly similar to that reported by Pavlic *et al.*

(2007). The cultural and morphological characters observed were exactly matching with the observations described by Norhayati *et al*. (2016).

Two isolates of fungal pathogen (MVPLB and NENLB) causing leaf blight were obtained from the sampling survey in 5 districts. MVPLB and NENLB produced thin pale white mycelium which later changed to light brown coloured when grown in PDA medium showing radial type of growth pattern. The pathogen was fast grower completing 9 cm growth within 3 days of inoculation. Hyphae were septate, initially appeared hyaline which later turned to light brown after seven days and the branches arised at right angles below the septa. Dark brown sclerotia like bodies with irregular shape developed on the culture when it matures. These observations were in congruence with those of Sneh *et al*. (1991) and Lal and Kandhari (2009). Thus the isolates were confirmed to be *Rhizoctonia* sp.

Four isolates of fruit rot pathogens *viz* MDKFR, NELFR, PAZFR, MVPFR were isolated on PDA and characterized based on cultural and morphological characteristics. MDKFR was characterized by sub surface growth of mycelium in PDA medium with dull whitish grey colour which completed the growth by 12 days and alternate zones were clearly visible in the medium. The fungus produced septate brown coloured mycelium with abundant conidia production and the conidia were brown in colour with transverse and longitudinal septa (muriform) with an average size of 31.87 µm length and 12.34 µm width and a prominent beak of 0.97 µm length. These results were similar to the observations described by Nagrale *et al*. (2013) and Ploetz *et al*. (2003) suggesting that the pathogen is *Alternaria* sp.

The fruit rot pathogen isolates collected from Nelliyambathy and Muvattupuzha *i.e*. NELFR and MVPFR produced appressed, submerged to slightly fluffy growth of mycelium in PDA which is white coloured initially and later turned grey in colour. Full growth of these fungal were produced within a week in a Petri plate (9 cm diameter). The fungus produced cream coloured spore mass after two weeks inside the petri plate and the reverse side of the petri plate appeared dark grey coloured. The hyphae were hyaline initially later turned to greyish black. The conidia were hyaline, single celled having an oblong shape with round ends and oil globules were seen at the centre. The average size of the conidia ranges from 8.08 - 10.32 µm length

and 2.49 - 3.01 µm width. These cultural and morphological characters were in congruence with the observations of Jeffries *et al.* (1990), Wolcan and Larran (2000) and Peres *et al.* (2002) and the pathogen was confirmed to be *Colletotrichum sp.*.

The isolate of fruit rot pathogen PAZFR collected from Pazhaynnur produced pure white aerial mycelium in PDA and the growth pattern was in radial manner. The pathogen completed full growth in the petri plate within 4 days and sclerotial bodies were started to appear in the petri plate within 10 days as smooth, perfectively round with shiny surface and the colour was yellowish initially turning to dark brown. The reverse side of petri plate appeared dull white to yellow colour. These observations were similar to the observation of Kwon *et al.* (2009) and the pathogen was confirmed as *Sclerotium* sp.

Two isolates of bacterial wilt pathogen *viz.* MNPWt and KANWt were collected from Manjapra of Ernakulam district and Kannara of Thrissur district respectively during the purposive sampling survey. Both the isolates produced colonies after 48 h of incubation. The isolation of these isolates on nutrient agar showed smooth, round, off white colonies with mucoid appearance and fluidal texture. These observations were in close conformity with the observations of Stanford and Wolf (1917), Khetmalas (1984) and Tahat and Sijam (2010) who described the colonies of *Ralstonia solanacearum* as white, wet, shining, circular, raised and smooth. When grown on tetrazolium chloride medium showed typical fluidal white coloured colonies of bacteria with reddish pink centre. The colony characters were similar to that of *R. solanacearum* observed by Khan (1974). The bacterial cell morphology was observed using scanning electron microscope (Tescan Vega-3 LMU) at Central Instrumentation Laboratory, College of Veterinary and Animal Science, Mannuthy. Electron microscopic image revealed that the pathogenic isolates causing wilt was rod shaped bacterium and the size of the bacterium were in the range 1.14 to 1.9 µm. An optimum working distance of 4.99 mm and voltage of 10kV was standardised by trial and error method for taking high resolution image for the bacteria. The morphology of bacterial cells observed were in close proximity with the observations of Shobha (2002), Narasimha and Srinivas (2012) and Pawaskar *et al.* (2014). The isolates showed positive reaction towards starch hydrolysis, oxidase test, casein hydrolysis, urease test and citrate test and negative

reaction towards gelatin hydrolysis and indole production. These results were in line with the reactions shown *R. solanacearum* as described by Das and Chattopadhyay (1955), Hossain *et al.* (2007), Singh *et al.* (2015), Shekhawat *et al.* (2000), Sharma and Singh (2019) and Seleim *et al.* (2014).

The morphology of virus associated with leaf malformation symptoms of passion fruit was studied by taking electron photomicrographs and revealed the presence of typical flexuous rods of virus with a particle size of 527.36 nm. Based on these observation, it was confirmed that the virus belongs to *Poty virus*. Similar type of flexuous rod-shaped particles of size 780 nm indicating *Potyvirus* infection were identified in leaf adsorption preparations of passion fruit woodiness infected subjected to electron microscopic examinations and no other virus-like particles were found by Ochwo-Ssemakula (2012). After 4 days of inoculation, necrotic local lesions were developed on the inoculated leaves confirming the virus as mechanically transmissible and 100 per cent transmission rate was recorded. Yeturu *et al*. (2018) also described sap transmission of virus associated with mosaic, blistering, curling, and necrosis symptoms of passion fruit on *Cucurbita pepo* (squash) and observed typical vein clearing and blistering symptoms after 11 days of post inoculation (dpi). The cleft grafting performed using scions showing typical symptoms taken from infected plants on 6 months old root stock revealed that the virus can be transmitted by grafting and a total of 10 healthy root stocks were grafted with infected scions. Five grafts were successful and all the, successful graft unions resulted in cent per cent transmission of virus. Similar to this, Nattrass (1944) described a method for transmitting the virus of the 'woodiness' disease of passion fruit by means of single leaf grafts.

5.5 MOLECULAR CHARACTERISATION

Fungal and bacterial pathogens collected during the purposive sampling surveys were characterized by cultural, morphological and biochemical characteristics. The confirmation of the species level identification of these pathogens were carried out by genomic DNA isolation and amplification of a specific region of the genome. ITS region and 16S rRNA of the genome was amplified in the case of fungal pathogens and bacterial pathogens respectively.

The fruit rot (MDKFR) and leaf spot (MDKLS) isolate collected from Vellanikkara of Thrissur district were characterised to be *Alternaria* sp. based on morphological and cultural characteristics. In order to identify the pathogen at species level, ITS sequencing was followed. Amplification of the ITS region of VKALS 2 was obtained at annealing temperature of 51.5 and 52.5° C. The nucleotide sequence of ITS region of isolate VKALS 2 retrieved from Agri genome, Kochi were analysed using nucleotide BLAST and it showed sequence similarities with the reported ITS gene sequences of *Alternaria porri.* The isolate showed similarity (99.82%) with 100 per cent query coverage and maximum score of 1050 with accessions MK905449.1 and KU504325.1.

For the species level identification of the isolate MNPLS, ITS sequencing was performed and the nucleotide sequence retrieved was analysed by comparing with the other nucleotide sequences available in NCBI and revealed that there is sequence similarity (of 94.72%) with 64 per cent query coverage and a maximum score of 1046 with accession LC074359.1 of *Lasiodiplodia theobromae*. The sequence also showed a similarity (91.49%) with accession JX282407.1 of the same. Other accessions with similarity are MT497430.1 (91.23%), KX965601.1 (94.4%), MK530029.1 (97.91%) and MK530038.1 (97.90%).

Leaf blight pathogens (MVPLB and NENLB) were characterised to be *Rhizoctonia* sp. based on cultural and morphological characteristics. The characterisation of MVPLB was carried out to find the species. Amplification of ITS region was carried out and was obtained at the annealing temperature of 53.7 and 53.9° C. The isolate showed similarity (99.73%) with 100 per cent query coverage and maximum score of 1347 with accessions HG934415.1 of *Rhizoctonia solani* and a similarity (99.59%) with 99 per cent query coverage and maximum score of 1339 with HG934419.1, another accession of same pathogen.

The sequence comparison of PAZFR with other sequences of NCBI showed a sequence similarity of 98.28 with accessions of *Athellia rolfsii* (MN872304), 98.14 (MN622806), 98.54 (MN610008) and 98.54 (MN610006) per cent with query coverage of 65, 66, 64 and 64 per cent respectively. The sequence also showed similarity of more than 98 per cent with other accessions like MN610004, MN610002, MN610000,

MK424482 and MH542664 confirming the pathogen to be *Athellia rolfsii* (*Sclerotium rolfsii*).

Six fungal leaf spot isolates (VKALS, NELLS, NENLS, MVPLS, AMBLS and THNLS) and two fungal fruit rot isolates (NELFR and MVPFR) were characterised to be *Colletotrichum* sp. based on cultural and morphological characteristics. The species level identification of all the *Colletotrichum* isolates were subjected to molecular characterisation by ITS sequencing. Comparison of nucleotide sequence of NELLS with the sequences available in NCBI revealed that the sequence showed a similarity (of 96.84%) with three accessions of *Colletotrichum brevisporum* namely KX463009, KU662423 and KP734236. Sequence comparison of NELFR showed that the sequence of NELFR has similarity of cent per cent with the accessions of *Colletotrichum brevisporum i.e.* MT560321, MT012108, MT232983, MH063256, MG525134, MG525133, KY986893, JN943068, JN943071. The nucleotide sequence of ITS region of MVPLS isolated from leaf spot sample of Muvattupuzha of Ernakulam district and MVPFR isolated from the fruit rot sample of the same location were subjected to NCBI BLASTn and the comparison with the available sequences in the database revealed 98.92 per cent similarity with a query coverage of 60 per cent and 97.53 per cent similarity with a query per cent of 74 per cent respectively with the accessions of *Colletotrichum brevisporum* namely MT012108 and MH063256. The sequence of ITS region retrieved for the isolate VELLS collected from the leaf spot samples of Vellanikkara, Thrissur district when compared with the nucleotide sequence of ITS showed 97.72 per cent similarity with a query coverage of 58 per cent with the ITS region of *Colletotrichuim brevisporum* belonging to the accessions MT560321. Several hits obtained by the sequence comparison of the isolate AMBLS collected from Ambalavayal of Wayanad showed a similarity of 99.57 per cent with a query coverage of 56 per cent with *Colletotrichum brevisporum* accessions namely MT560321, MT012108, MG461529, KT949407, KR534660, KC815123, GU066694, GU066653 and FJ233190. The sequence comparison of the isolate, THNLS collected from Thankamani of Idukki district showed a similarity of 100 per cent with a query coverage of 62 per cent with *Colletotrichum brevisporum* belonging to the accessions MT560321, MT012108, MT232983, MH063256, MG525134, MG525133, KY98689,

JN943068, JN943071 and MT361074. The sequence retrieved for the isolate NENLS, when analysed showed a similarity of 99.40 per cent with *Colletotrichum queenslandicum* belonging to the accessions MN547511, MK119210 and MF380919. These results confirmed that the major pathogen responsible for anthracnose of leaves and fruit of passion fruit in Kerala as *Colletotrichum brevisporum.* Similar results were obtained by Du *et al.* (2017) while dealing with the anthracnose of passion fruit in China. *Colletotrichum queenslandicum* was also recorded in passion fruit by James *et al.* (2014) while dealing with the postharvest diseases of passion fruit.

Multiple sequence analysis was done with all these eight isolates of *Colletotrichum* and by using the bioinformatics tool, ClustalW the relationship among these isolates was studied. Phylogenetic tree showed that all these eight isolates shared a common ancestor. The *Colletotrichum* isolates collected from passion fruit form two clusters indicating species diversification. The isolates NELFR, MVPLS, VELLS, AMBLS, NENLS and NENLS) formed a single cluster and the isolates THNLS and MVPFR formed another cluster indicating some diversity within species. A neighbouring-joining phylogenetic tree made with four representative isolates (NELLS, MVPLS, AMBLS and VELLS) of *Colletotrichum brevisporum* collected from the study and other *Colletotrichum* (six *C. brevisporum* accessions, four *C. capsici* and four *C. gloeosporoides*) accessions reported from India and elsewhere available in the GeneBank database revealed that the collected isolates of *Colletotrichum brevisporum* and the accessions of *Colletotrichum brevisporum* formed a single group. The accessions of *Colletotrichum gloeosporoides* and *Colletotrichum capsici* formed sub groups of each in a second group. These two groups shared a common ancestor. In the phylogenetic tree constructed by Du *et al*. (2017) based on ITS sequences, the two representative isolates were placed within a clade comprising two reference isolates of *C. brevisporum* and this clade formed a subgroup with another clade of *C. gloeosporoides.*

Based on cultural, morphological characters and biochemical characters the pathogen associated with wilt (MNPWt and KANWt) collected from Mnajapra and Kannara was identified as *Ralstonia solanacearum*. The identity of the pathogen was confirmed at the molecular level by amplification of 16 S rRNA region of the pathogen.

The amplification of 16S rRNA region of *Ralstonia solanacearum,* sequencing and *in silico* analysis revealed that the sequence showed maximum homology of 97 per cent with *R. solanacearum* (Accession no. MF973211). Thus, confirmed the bacterial wilt pathogen of passionfruit as *Ralstonia solanacearum*. Lopes *et al.* (1999) described passion fruit as one of the host for *Ralstonia solancearum,* the wilt pathogen.

5.6 CORRELATION OF WEATHER PARAMETERS WITH PER CENT DISEASE SEVERITY

Weather parameters like rainfall, relative humidity, maximum and minimum temperature prevailed in each location during the period of survey were collected and the preliminary analysis revealed that the rainfall was higher in the place with highest severity of fungal disease. Therefore, correlation analysis was done with the values of per cent disease severity of fungal disease and the weather parameters.

Conducive environmental conditions were inevitable for disease development and is an important component in disease triangle. Correlation analysis revealed that rainfall pattern of the location has a strong positive correlation with the severity of fungal diseases and the correlation coefficient was found to be 0.895. Highest PDS for the fungal diseases was observed in Muvattupuzha region of Ernakulam district *i.e.* 58.73 per cent and 63.90 per cent for the leaf spot disease (MVPLS) and leaf blight (MVPLB) where the amount of rainfall was also higher. Similarly, relative humidity has also positive correlation with the severity of fungal diseases where the correlation coefficient is 0.795. Therefore, when the relative humidity is higher, the severity of fungal disease is high. When the effect of temperature on severity of disease was analyzed, it has a negative correlation with the severity of the disease and have a correlation coefficient of (–) 0.868. All these observations were in line with the observations of Piza Jr (1994), Ploetz *et al.* (2003) and Junquiera *et al.* (2005).

5.7 DISEASE MANAGEMENT

Chemical control is one of the inevitable approach in integrated disease management. There exists a gap between the need for resistant cultivars against various diseases and their availability. Hence, use of fungicides is the one of the better options available when a pandemic outbreak occurs. The fungicides need to be applied

judiciously against various diseases. Wide range of fungicides and bicontrol agents are available in the market and this necessitates the need for *in vitro* evaluation of these measures against the major pathogens identified in passion fruit for fixing the spraying schedule for *in vivo* application.

In vitro evaluation of fungicides against one major foliage pathogen (*Colletotrichum brevisporum*) and one fruit rot pathogen (*Sclerotium rolfsii*) was carried out. Seven fungicides at three different doses (Copper hydroxide (0.05, 0.1, 0.2%), Hexaconazole (0.1, 0.2, 0.3%), Propineb (0.15, 0.25, 0.35%), Difenoconazole (0.025, 0.05, 0.075%), Carbendazim 12% + mancozeb 64% (0.1, 0.2, 0.3%), Cymoxanil 8% + mancozeb 64% (0.1, 0.25, 0.5%), Azoxystrobin (0.005, 0.01, 0.015%)) and 1 per cent Bordeaux mixture was used for poison food technique.

Copper hydroxide, hexaconazole and a combination of carbendazim and mancozeb were highly effective against *Colletotrichum brevisporum* when applied at all the three doses *i.e.* higher, recommended and lower doses. Cent per cent inhibition was noticed in all these cases and the treatment effect is on par. Amata *et al.* (2013) described similar observations where Carbendazim and hexaconazole were the most effective treatment while dealing with die back disease of passion fruit caused by *Colletotrichum* sp. Another study by Dev and Narendrappa (2016) also given results in congruence with the effectiveness of carbendazim and hexaconazole against *Colletotrichum* sp. The effectiveness of triazole group of fungicides was explained by the interference of this group of fungicides with the biosynthesis of fungal sterols and inhibit the ergosterol biosynthesis. Ergo-sterol is essential to the structure of cell wall and its absence cause irreparable damage to cell wall leading to death of fungal cell. A similar study was reported for the effectiveness of Triazoles, which inhibit the sterol biosynthesis pathway in fungi (Nene and Thapliyal, 1973). Prashanth (2007) reported that among four systemic fungicides maximum % inhibition of growth of *C. gloeosporioides* was observed in Difenconazole (90.78 %) and Propiconazole (90.78 %), Gud and Raut (2008) reported that Thiophenate-methyl and Propiconazole were most effective against *C. gloeosporioides* followed by Hexaconazole and Carbendazim. Patel (2009) and Pandey *et al.* (2012) studied that among the tested fungicides Tricyclazoles were found to be superior for controlling *C. gloeosporioides* causes

anthracnose. The fungicides, propineb and azoxystrobin showed cent per cent inhibition at recommended and higher doses.

Two fungicides *i.e.* Azoxystrobin (0.005, 0.01, 0.015%) and Hexaconazole (0.1, 0.2, 0.3%) were cent per cent effective against *Sclerotium rolfsii* at lower, recommended and higher doses. Similar results with respect to complete inhibition of *Sclerotium rolfsii* by Hexaconazole were given by Chowdhary *et al.* (1998), Arunasri *et al.* (2011) and Manu *et al.* (2012) and by Shirsole *et al.* (2019) with respect to Azoxystrobin. The effectiveness of strobilurin fungicide, Azoxystrobin was explained by its capability to inhibit mitochondrial electron transfer by binding to the Qo center of cytochrome bc_1 complex and interfering with ATP synthesis (Ulrich *et al.* (1988); Ypema and Gold (1999); Xiao *et al.* (2002) and Esser *et al.* (2004). Complete inhibition of mycelial growth of *Sclerotium rolfsii* was shown by the combination of cymoxanil and mancozeb (Curzate) at higher dose of 0.5 per cent but at recommended dose of 0.25% per cent the fungicide showed only 80.29 per cent inhibition. Similarly, the combination of carbendazim and mancozeb produced only 43.66 per cent and 77.44 per cent inhibition when applied at lower (0.1%) and recommended dose (0.2%). But at a higher dose of 0.3 per cent, it gave cent per cent inhibition of the pathogen. Therefore, the combination products containing Mancozeb was effective against *Sclerotium rolfsii* at higher doses and this observation was in congruence with the observations of Manu *et al.* (2012). Difenaconazole when applied at a lower dose of 0.025% resulted in 85.28 per cent, at recommended dose of 0.05 per cent resulted in inhibition of 88.89 per cent and complete inhibition was observed at higher dose of 0.075 per cent. The result was in accordance with those of Virupakshaprabhu and Hiremath (2003). An inhibition per cent of 75.28, 87.50 and 100 was observed when propineb was used at lower (0.15%), recommended (0.25%) and higher doses (0.35%) respectively. Shirsole *et al.* (2019) also revealed that propineb when applied at higher dose was effective against *Sclerotium rolfsii*. Copper hydroxide produced 85.83, 88.89 and 100 per cent inhibition when applied at three doses *i.e.* lower (0.05%), recommended (0.1%) and higher doses of 0.2 per cent respectively. Bordeaux mixture (1%) was not that effective against *Sclerotium rolfsii*, it only showed an inhibition less than 50 per cent *i.e.* 47.5 per cent.

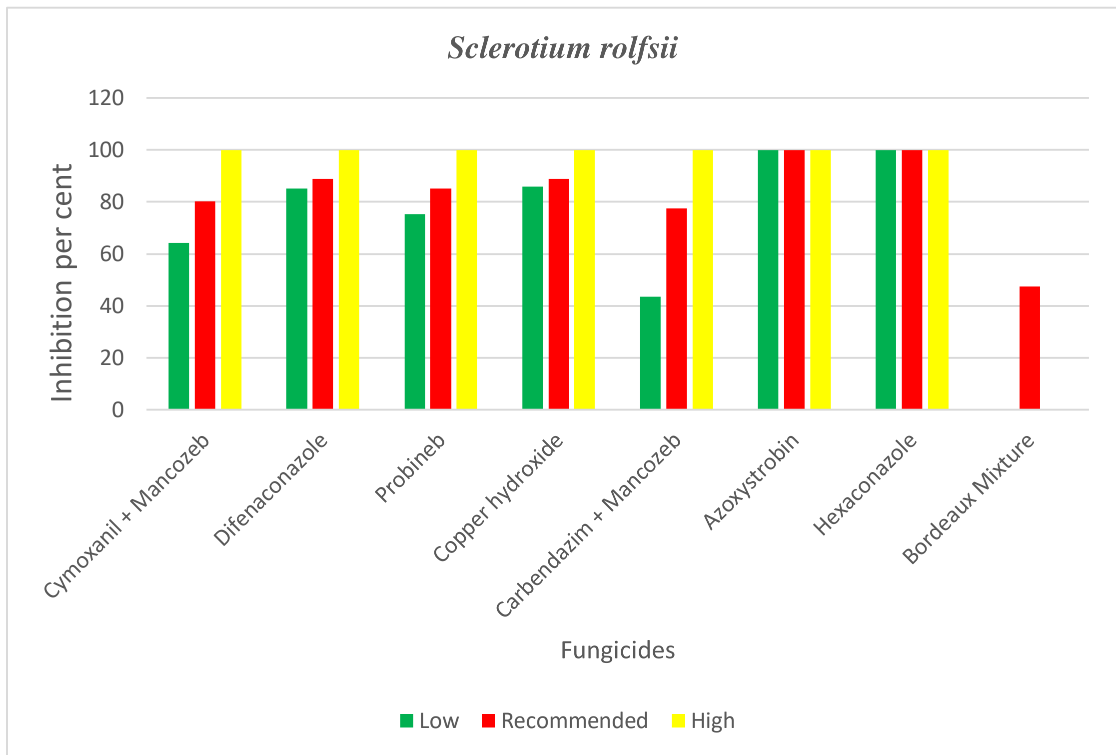


Fig 11a*: In vitro* evaluation of fungicides agents against *Sclerotium rolfsii*

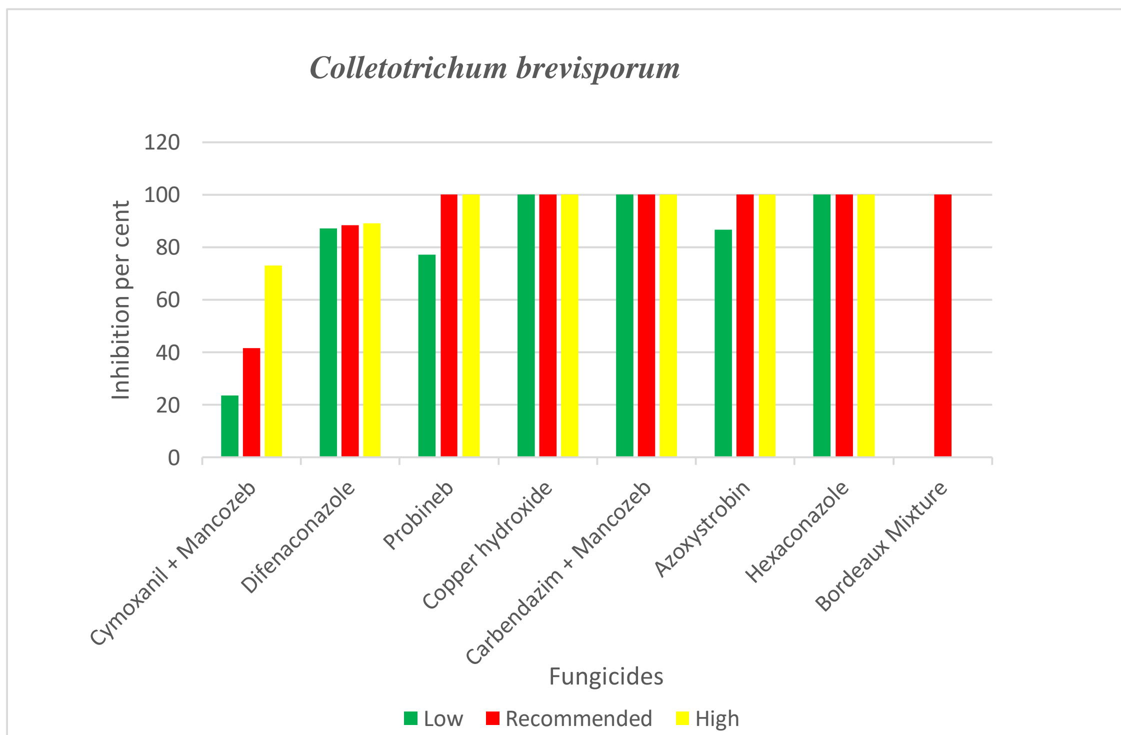


Fig 11b: *In vitro* evaluation of fungicides against *Colletotrichum brevisporum*

The *in vitro* evaluation of Bio control agents revealed that that *Trichoderma* sp. was capable of inhibiting the growth of *Sclerotium rolfsii* and *Colletotrichum brevisporum.* An inhibition of 50 per cent was observed for *Sclerotium rolfsii* by the antagonist *Trichoderma* sp. and inhibition of the growth of *Sclerotium rolfsii* was not observed when *Pseudomonas fluorescence* was used. This observation was in agreement with the observations of Bari et al. (2000), Kulkarni (2007), Basamma (2008) and Manu *et al*. (2012). When the same was used against *Colletotrichum brevisporum,* cent per cent inhibition was observed. *Pseudomonas fluorescence,* the bacterial antagonist produced an inhibition of 72.22 per cent when used against *Colletotrichum brevisporum.*

Summary

6. SUMMARY

Passion fruit is a high value fruit crop which is popular among health conscious people and have great export potential. Kerala farmers are showing much importance to the crop due to assured demand and climatic suitability. But the market value of the fruits is greatly reduced due to various diseases affecting the crop at different growth stages. Hence, the present study "Characterization and documentation of diseases of passion fruit (*Passiflora edulis* Sims.) was undertaken to identify the diseases prevailing in various passion fruit orchards of the state, characterize the pathogen associated with each disease and to study correlation of weather parameters, pathogenicity, symptomatology and *in vitro* management studies of major fungal pathogens.

Intensive sampling surveys were conducted in major passion fruit growing tracts of five districts *i.e.* Thrissur, Palakkad, Ernakulam, Wayanad and Idukki of the state which revealed the presence of leaf spots, leaf blights, fruit rots, wilting and leaf malformation in the crop. The symptoms collected during the survey were catalogued and expressed as leaf spots (VKALS, MDKLS, MNPLS, NELLS, NENLS, MVPLS, THNLS and AMBLS), leaf blights (MVPLB and NENLB), fruit rots (MDKFR, PAZFR, MVPFR and NELFR), wilting (MNPWt and KANWt) and leaf malformation (VKALM).

Two leaf spots (VKALS and MDKLS), one fruit rot (VKAFR), one wilt (KANWt) and one leaf malformation (VKALM) symptoms were collected from Thrissur district. Among the various symptoms collected from different fields of the district, maximum per cent diseases severity (PDS) was recorded for the fruit rot, PAZFR (53.59%) with an incidence of 26.47 per cent. The highest severity among the leaf spot symptoms was observed for VKALS (32.48%) with 18.47 per cent incidence followed by MDKLS (25%) with an incidence of 16.28 per cent. Even though the fruit rot sample (MDKFR) showed an incidence of 39 per cent, the severity was only 13 per cent. The wilting symptom (KANWt) showed an incidence of 33 per cent and the affected plant was completely lost due to infection.

From Ernakulam district, four different symptoms were obtained of which two were leaf spots, one was leaf blight and another was wilt. Among the samples collected from Ernakulam district, the highest per cent disease severity (63.90%) and incidence (78%) was shown by the leaf blight sample (MVPLB) followed by leaf spot, MVPLS (PDS-58.73% and PDI – 43%). Wilting of the entire vines of passion fruit (MNPWt) was found to be more severe in Manjapra of Angamaly region with an incidence of 37.64 per cent in which the entire plant was lost and resulted in huge economic loss to the farmers. The lowest severity (of 9.25%) was observed for the leaf spot isolate, MNPLS collected from the same region with an incidence of 14.80 per cent.

The passion fruit orchards from Nelliyampathy and Nemmara were selected as the two locations for the purposive sampling survey from Palakkad district. Two leaf spot samples (NELLS and NENLS), one leaf blight (NENLB) and one fruit rot sample (NELFR) were collected with per cent disease severity of 38.53, 35.49, 39.66 and 36.76 respectively. Among these samples collected, NENLB showed highest incidence of 95 per cent followed by NELLS (88.75%), NELFR (78.25%) and NENLS (75%). Leaf spot samples were only obtained from Idukki and Wayanad districts. Two isolates *i.e.* AMBLS and THNLS were observed with a severity of 40.23 per cent and 40.84 respectively. These samples showed incidence of 48.9 (AMBLS) and 39.92 (THNLS) per cent.

The weather parameters of the surveyed locations were collected from the website of Marksim and correlation analysis was carried out with the Per cent disease severity (PDS) of fungal diseases. Preliminary analysis revealed that the leaf spots, leaf blights and fruit rot symptoms collected were due to pathogenic fungi. The analysis revealed that, rainfall pattern has a strong positive correlation with the severity of fungal diseases and the correlation coefficient is found to be 0.895**. Highest PDS for the fungal diseases was observed in Muvattupuzha region of Ernakulam district *i.e* 58.73 per cent and 63.90 per cent for the leaf spot disease (MVPLS) and leaf blight (MVPLB) where the amount of rainfall was also higher. Relative humidity has also shown a positive correlation with the severity of fungal diseases with a correlation coefficient of 0.795**. Therefore, when the relative humidity is higher, chance of development of fungal disease with high severity is more. Temperature of the location has a negative

correlation with the severity of the disease and have a correlation coefficient of (–) 0.868**.

The pathogenicity of different pathogens (fungal and bacterial) isolated from the passion fruit were established by following Koch's postulates under *in vitro* conditions by artificial inoculation. The symptoms under artificial inoculation were studied for each isolates and the symptoms developed were found similar to those observed under natural conditions. Under natural condition, VKALS produced small oval shaped light coloured spots which later turned to brown colour with prominent yellow halos around and acervuli at the center. MDKLS were characterized by small minute brown spots of 0.3 x 0.5 cm size distributed throughout the leaf lamina with clear concentric zonation at later stages. In the case of MNPLS, irregular shaped spots of white colour were produced with a papery texture and later the infected areas fall giving shot hole appearance. The symptoms of MVPLS were characterized by large spots in the marginal areas of leaves with 2.00 cm size followed by drying of leaves from the marginal areas were observed which later produced necrotic patches with fruiting bodies on leaf lamina. NELLS produced numerous small irregular spots initially which later coalesced to form necrotic patches with acervuli at the advanced stages of disease and severe chlorosis around the infected area. Symptoms of NENLS initially appeared as white coloured spots with brown margin which later turned to brown spots with yellow halo around them and mainly seen in the interveinal areas of the leaves. The isolate of leaf spot pathogen from Ambalavayal (AMBLS) produced creamy white coloured light spots with yellow halos around the spots and light brown margins were found to delimit the spots. In the case of THNLS, minute light coloured spots were produced which later enlarged and the spots coalesced to produce extensive blighted areas in the leaf lamina and yellow halos were also seen around the spots. Symptoms of MVPLB, the leaf blight sample from Muvatttupuzha produced cream coloured small spots of about 0.1 - 0.3 cm size initially and later these spots enlarged and gave rise to irregular blighted appearance in the leaves with a uniform distribution throughout the leaf lamina. In the case of NENLB, the leaf blight sample from Nemmara produced extensive blighted areas in the interveinal areas with light colour and papery texture and having a brittle nature.

Four isolates causing fruit rots *i.e.* VKAFR, NELFR, PAZFR and MVPFR were collected by conducting the sampling surveys in five districts of the state. VELFR, produced light coloured sunken areas and the spots were not water soaked rather dry with fructifications of fruiting bodies inside the spots. The fruit rot sample collected from Nelliyampathy (NELFR) were characterized by dark brown coloured spots on the mature fruits which later turning to soft and sunken rotten areas. PAZFR produced necrosis on the fruits and the initial symptom appeared as light pink coloured rotten areas with white cottony mycelial growth which later extended to produce extensive rotting of the fruits affecting the fruit pulp. Sclerotial bodies were formed on the advanced stages of the diseases. The fruit rot sample collected from Muvattupuzha of Ernakulam district (MVPFR) was characterized by symptoms such as light coloured spots which later enlarged to produce larger creamy white spots ultimately leading to the rotting of the fruits and black spore masses were seen at the centre of spots on the advanced stages.

Two isolates of wilt causing pathogen were obtained from the wilt samples. Sudden wilting of the entire vines of passionfruit was observed as the initial symptom. The plants of all stages were infected but more incidence was observed in fruiting plants. This highlights the importance of the disease which leads to complete yield and crop loss to the farmers. The most prominent symptom was the rapid wilting of the entire vines while the leaves retained its green colour. Within few days of infection, the entire plant was wilted and dried spreading the infection to nearby vines. The other symptoms found associated with the diseases are discolouration of vascular bundles and extensive rotting of roots.

Symptoms similar to the infection caused by virus were seen in Vellanikkara (VKALM) and the symptoms include: islands of light green and dark green areas, vein clearing, extreme reduction of leaf lamina, leathery appearance of leaf, puckering and cupping of leaf, malformed fruits and chlorotic spots.

The fungal pathogens were identified up to the genus level by cultural and morphological characters. It was confirmed that the six leaf spots (VKALS, NELLS, NENLS, MVPLS, THNLS and AMBLS) and two fruit rots (MVPFR and NELFR) were caused by *Colletotrichum* sp.. The fungal genus responsible for leaf blights (MVPLB

and NENLB) were *Rhizoctonia* sp.. *Alternaria* sp. was responsible for the leaf spot (MDKLS) and fruit rot (MDKFR). MNPLS was caused by *Diplodia* sp. and PAZFR was due to *Sclerotium* sp. The pathogenic isolates causing wilt (MNPWt and KANWt) were characterized by morphological, cultural and biochemical characters and found to be a gram negative, rod shaped (by Scanning Electron microscopic analysis) bacterium. The cultural characters on TZC medium and biochemical tests performed revealed it as *Ralstonia solanacearum*. The virus associated with leaf malformation (VKALM) were characterized biologically and found to be transmissible by mechanical inoculation as well as grafting. The Transmission Electron microscopic analysis of symptomatic leaves revealed the presence of typical flexuous rods of virus particles with a size of 527.36 nm. Based on these observations, the virus associated with leaf malformation was confirmed to be *Potyvirus*.

The species level identification of the pathogens was carried out by molecular characterisation using ITS primers (in case of fungal isolates) and primers specific to 16S rRNA (in case of bacterial pathogen). The sequences retrieved were analysed using NCBI Blast and the *in silico* analysis revealed that the *Colletotrichum brevisporum* was responsible for five leaf spots (VKALS, NELLS, NENLS, MVPLS, THNLS and AMBLS) and two fruit rots (MVPFR and NELFR). *Colletotrichum queenslandicum* was the pathogen associated with NENLS. *Alternaria porri* was the causal agent of the leaf spot (MDKLS) and the fruit rot (MDKFR). The leaf blight isolates from MVPLB and NENLB were *Rhizoctonia solani. Lasiodiplodia theobromae* was the causal agent of MNPLS and *Sclerotium rolfsii* was the causal agent of PAZFR. The wilt pathogen associated with MNPWt and KANWt was confirmed to be the bacterium, *Ralstonia solanacearum*. Molecular diagnosis of *Potyvirus* associated with leaf malformation of passion fruit was done using reported primers specific to the Cylindrical inclusions (CI) protein of the viral genome. An amplicon of expected size (680 bp) was produced which confirmed the association of *Potyvirus* as the causal agent of leaf malformation of passion fruit.

In vitro evaluation of eight fungicides and two bio control agents were carried out against a major foliar pathogen (*Colletotrichum brevisporum*) and a fruit rot pathogen (*Sclerotium rolfsii*). Two fungicides *i.e.* Azoxystrobin (0.005, 0.01, 0.015 %)

and Hexaconazole (0.1, 0.2, 0.3%) were cent per cent effective against *S. rolfsii* at lower, recommended and higher doses. Copper hydroxide, hexaconazole and a combination of carbendazim and mancozeb were highly effective against *C. brevisporum* at all the three doses *i.e.* higher, recommended and lower doses and cent per cent inhibition was observed.

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Appendices

Appendix I

Composition of media for isolation of pathogens (fungi and bacteria) from symptomatic samples

1. **Potato dextrose agar (PDA)**

   Potato – 200 g

   Dextrose – 20 g

   Agar – 20 g

   Distilled water – 1000 ml

2. **Nutrient agar (NA)**

   Peptone – 20 g

   Beef extract – 1 g

   Na Cl – 5 g

   Agar – 20 g

   Distilled water – 1000 ml

   pH – 6.5 – 7.5

Appendix II

Composition of media for cultural characterisation of bacteria

Tetrazolium chloride (TZC) medium

TZC Basal medium-

Peptone – 10 g

Casein hydrolysate – 1 g

Glucose – 5g

Agar – 20 g

Distilled water – 1000 ml

pH – 6.8

Dissolve 1 g of TZC in 100 ml of distilled water separately and placed in a light proof capped bottle and autoclaved for eight minutes. From this stock solution, 5 ml was added to each 1000 ml basal medium.

Appendix III

Composition of media for biochemical characterisation of bacterial pathogens

1. **Starch agar medium**

   Soluble starch – 20g

   Peptone – 5g

   Beef extract – 3g

   Distilled water – 1L

   pH – 7

2. **Nutrient gelatin agar medium**

   Peptone – 5g

   Beef extract – 3g

   Gelatin – 120 g

   Distilled water – 1L

   pH – 6.8

3. **Skim milk agar medium**

   Skim milk powder – 100g

   Peptone – 5g

   Agar – 20g

   Distilled water – 1L

   pH- 7.2

4. **Urea agar medium**

   Peptone – 1g

   NaCl – 5g

   KH_2PO_4 – 2g

   Agar- 20g

   Distilled water – 1L

   pH – 6.8

5. **Tryptone broth (1%)**

   Peptone – 10g

Distilled water – 1L

6. **Simmond's citrate agar**

Ammonium dihydrogen phosphate – 1g

Dipotassium phosphate- 1g

NaCl – 5g, sodium citrate- 2g

Magnesium sulphate- 0.2g

Agar - 15g

Bromothymol blue – 0.8g

Distilled water – 1L

Appendix IV

Composition of buffer for mechanical transmission

0.1 M Potassium phosphate buffer (pH: 7)

Prepare 1M each of dipotassium hydrogen phosphate (K_2HPO_4) and potassium dihydrogen phosphate (KH_2PO_4).

Combine 61.5 ml of 1M K_2HPO_4 and 38.5 ml of KH_2PO_4. Dilute the combined 1M stock solution to 1 litre with distilled water. Adjust the pH TO 7.0 with pH meter.

Appendix V

Composition of buffers and gels for molecular characterization of pathogens

1. **Extraction buffer (2% CTAB)**

Tris (100 Mm) - 1.2114 g

EDTA (20 Mm) - 0.7448 g

NaCl (1.4 M) – 8.1816 g

CTAB – 2 g

Distilled water – 100 ml

pH – 8

2. **CTAB solution (10%)**

CTAB – 10 g

NaCl (0.7 M) – 4.1 g

Water – 100 ml

3. **Electrophoresis buffer (50X TAE)**

Tris base – 242 g

Glacial acetic acid – 57.1 ml

0.5 M EDTA (pH 8) **-** 100 ml

Distilled water: 1000 ml

Appendix VI

Accession numbers of the sequences deposited in Genebank

Pathogen	**Accession number**
Alternaria porri	MT830901
Colletotrichum queenslandicum	MW055667
Diaporthe phaseolorum	MW055668
Rhizoctonia solani	MW057250
Colletotrichum brevisporum	waiting
Sclerotium rolfsii	waiting
Lasiodiplodia theobromae	waiting
Ralstonia solanacearum	waiting

Appendix VII

Details of accessions related in the phylogenetic analysis of pathogens

Pathogen	Related accession	Details	
		Host	**Location**
Alternaria porri	MK905449	Banana	Karnataka
Lasiodiplodia theobromae	MT103322	Pine tree	Mexico
Rhizoctonia solani	HG934415	Balsam	China
Sclerotium rolfsii	MN610004	Mung bean	China
Diaporthe phaseolorum	HM012819	Mangroves	Thailand
Ralstonia solanacearum	KP017459	Chilli	Karnataka

CHARACTERIZATION AND DOCUMENTATION OF DISEASES OF PASSION FRUIT
(*Passiflora edulis* Sims.)

By

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ABSTRACT OF THE THESIS

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ABSTRACT

Passion fruit (*Passiflora edulis* Sims.), a native of Brazil is an emerging high value crop with fruits of great export potential. It is a woody, climbing vine which is valued for its fruits with unique flavor and aroma as well as for its nutritional and medicinal properties. During the recent past, there is tremendous increase in the cultivation of passion fruit, particularly in the state of Kerala. This is because of its importance as an assured source of income to small and marginal farmers. But, the longevity and productivity of passion fruit vines are often challenged by many biotic stress especially diseases. Even though many diseases are reported internationally, no systematic study has been conducted in the country particularly in Kerala due to the recent introduction of this crop in the agricultural scenario of the state. Hence, the present study was undertaken to identify the diseases affecting passion fruit occurring in Kerala, documentation of the same and characterization of associated pathogens.

Purposive sampling surveys were conducted in the passion fruit growing tracts of five districts *viz.* Thrissur, Ernakulam, Palakkad, Wayanad and Ernakulam during the period of March, 2019 to January, 2020. Eight leaf spots (VKALS, MDKLS, NELLS, NENLS, MNPLS, MVPLS, THNLS and AMBLS), two leaf blights (NENLB and MVPLB), four fruit rots (MDKFR, NELFR, MVPFR and PAZFR), one bud blight (KLYBB), two wilts (MNPWt and KANWt) and one leaf malformation (VKALM) were observed during the survey. Among the foliage diseases, leaf blight (MVPLB) recorded the highest per cent disease incidence (PDI) of 78 per cent with a severity of 63.9 per cent. Among the fruit diseases, PAZFR recorded the highest incidence of 53.59 per cent disease with a severity of 26.47 per cent. The disease incidence of wilt; MNPWt and KANWt were 37.64 and 33 per cent respectively.

Isolation and pathogenicity studies of the associated microorganisms yielded 15 fungal isolates (leaf spots, leaf blights and fruit rots), two bacterial isolates (wilt) and one virus isolate (leaf malformation). Symptoms of the different diseases were studied both under natural and artificial conditions. The fungal isolates were characterized and identified by studying the cultural and morphological characteristics. Based on these characters, *Colletotrichum* sp., *Alternaria* sp. and *Lasiodiplodia* sp.

were identified as leaf spot pathogens, *Rhizoctonia* sp. as leaf blight pathogen, *Phomopsis* (*Diaporthe*) sp. as the bud blight pathogen and *Colletotrichum* sp., *Alternaria* sp. and *Sclerotium* sp. as fruit rot pathogens infecting passion fruit. The bacterial isolates (MNPWt and KANWt) causing wilt disease in passion fruit was identified as *Ralstonia solanacearum* Yabuuchi *et al.* based on cultural, morphological and biochemical characters. The differential host inoculation test conducted for race identification of the bacteria revealed that it belongs to Race 1. Morphological and biological characterisation of virus associated with leaf malformation revealed the association of *Potyvirus* as the causal organism of leaf and fruit malformation.

Molecular characterization was carried out by genomic DNA isolation and amplification of specific region of fungal genome by Polymerase Chain Reaction (PCR) for the species level identification of the isolates. Amplification of Internal Transcribed Spacer (ITS) region of the fungal isolates followed by ITS sequencing and *in silico* analysis confirmed that the pathogen causing VKALS, NELLS, MVPLS, THNLS, AMBLS, NELFR and MVPFR as *Colletotrichum brevisporum,* MDKLS and MDKFR as *Alternaria porri*, NENLS as *Colletotrichum queenslandicum,* MNPLS as *Lasiodiplodia theobromae*, MVPLB and NENLB as *Rhizoctonia solani,* KLYBB as *Diaporthe phaselorum* and PAZFR as *Sclerotium rolfsii*. The bacterial isolates (MNPWt and KANWt) causing wilt disease in passion fruit was further confirmed as *R. solanacearum* through the amplification of 16 S rRNA region of the genome followed by sequencing and *in silico analysis*. The molecular characterization of *Potyvirus* associated with passion fruit was carried out using RNA extraction followed by cDNA synthesis and amplification of gene corresponding Cylindrical Inclusion (CI) protein using degenerate primers (Ha *et al*., 2008). The PCR yielded the expected band size of 680 bp, thus confirming *Potyvirus* as the causal organism of fruit and leaf malformation.

An *in vitro* experiment was conducted to study the efficacy of fungicides and biocontrol agents against major fungal pathogens *viz. C. brevisporum* and *S. rolfsii.* Fungicides *viz.* copper hydroxide, hexaconazole, propineb, difenoconazole, carbendazim 12% + mancozeb 64%, azoxystrobin and Bordeaux mixture were found to be very effective against *C. brevisporum* at recommended dose and showed cent per

cent inhibition of fungal growth. In the case of *S. rolfsii*, fungicides *viz.* hexaconazole and azoxystrobin were found to be effective even at a lower dose. Dual culture studies revealed that the biocontrol agent, *Trichoderma* sp. was effective against both the pathogens *viz. C. brevisporum* and *S. rolfsii* whereas *Pseudomonas fluorescens* was effective only against *C. brevisporum.*