PHYLLOSPHERE MICROORGANISMS FOR THE MANAGEMENT OF ANTHRACNOSE DISEASE OF COWPEA (*Vigna unguiculata* (L.) Walp.).

By

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(2019-11-108)

THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT PATHOLOGY)

Faculty of Agriculture

Kerala Agricultural University

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE

VELLANIKKARA, THRISSUR – 680656

KERALA, INDIA

2022

DECLARATION

I, Anupama P.R. (2019-11-108) hereby declare that the thesis entitled **"Phyllosphere microorganisms for the management of anthracnose disease of cowpea (*Vigna unguiculata* (L.) Walp.)"** is a bonafide record of research work done by me during the course of research and the thesis has not previously formed for the award of any degree, diploma, fellowship or other similar title, of any other university or society.

Place: Vellanikkara

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Certified that the thesis entitled **"Phyllosphere microorganisms for the management of anthracnose disease of cowpea (*Vigna unguiculata*(L.) Walp.)"** is a record of research work done independently by Ms. Anupama P.R.(2019-11-108) under my guidance and supervision and that it has not previously formed the basis for the award of any degree, diploma, associationship or fellowship to her.

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ACKNOWLEDGEMENT

First and foremost, I would like to bow my head before the ***ALMIGHTY GOD*** *for giving me strength and encouragement throughout all the challenging moments of this research programme.*

With immense pleasure I avail this opportunity to express my deep sense of gratitude and reverence to my major advisor, ***Dr. Sainamole Kurian P.****, Professor and Head, Dept. of Plant Pathology, College of Agriculture, Vellanikkara. Her valuable guidance and encouragement helped me in all the time of research and above all, the extreme patience, understanding and whole hearted co-operation rendered throughout the course of my study. Without her support, esteemed advice and immense help completion of this research could not have been possible. I always remain indebted to her.*

I express my gratitude to ***Dr. Reshmy Vijayaraghavan****, Assistant Professor, Dept. of Plant Pathology, College of Agriculture, Vellanikkara, for her timely help and suggestions throughout the research programme.*

I snicerely express my gratitude to ***Dr. Gleena Mary C.F****,, Assistant Professor, Dept. of Plant Pathology,College of Agriculture, Vellanikkara, for her valuable suggestions and support throughout the research programme.*

I express my sincere gratitude and indebtedness towards ***Dr. Rashmi C.R.****, Assistant Professor, AICVIP, Dept. of Vegetable Science,College of Agriculture, Vellanikkara and a member of my advisory committee for her valuable guidance and constant encouragement throughout the research programme and I'm always remain indebted to her.*

*I express my gratitude towards **Dr. Surendra Gopal**, Professor and Head, Dept. of Agricultural Microbiology, College of Agriculture, Vellanikkara for his critical evaluation, expert advice during my research work.*

*I express my heartfelt thanks to **Dr. Anita Cherian K.,** (Retd.), for her valuable suggestions and comments during my research work.*

*I express my gratitude towards **Dr. Chitra B Nair**, Assistant Professor, Dept. of Plant Pathology, College of Agriculture, Vellanikkara, for her timely support throughout the research programme.*

*I express my gratitude towards**Dr. Anju and Dr. Shayida**, Assistant Professors, Dept. of Plant Pathology, College of Agriculture, Vellanikkara, for their valuable suggestions and support throughout the research programme.*

*I'm extremely grateful to **Dr. Dicto Jose**, Assistant Professor, Agricultural Research Station, Mannuthy, for his valuable guidance and constant encouragement throughout the research programme.*

*I extremely thankful to **Dr. Rehna Augustin**, Assistant Professor, Dept of Plant Biotechnology, College of Agriculture, Vellanikkara, for her expert advice and valuable suggestions throughout the research programme.*

*I would like express my special thanks to **Milsha chechi, Ananthu ettan, Anjali chechi, Sharon chechi, Karthika chechi, Liji chechi, Neethu chechi, Prasanna chechi, Fridin chechi** and **Dilna chechi** for the help and support rendered during my work in the Department.*

*With deep reverence, I express my heartfelt thanks and never ending indebtedness to my classmates **Arya, Nitha, Sujisha, Gayathri, Likhita, Sowmya** for*

their unbounded love, motivation and support which led to the successful completion of my research work. I owe thanks to ***Vyshu, Achu, Chiru, Sandra, Anulatha, Arya, Ankitha, Swathi*** *and* ***Sreelakshmi*** *whose support and help throughout the PG programme could never be forgotten.*

My heartfelt thanks to my seniours ***Athing Didi, Laya chechi, Ajayasree chechi, Aparna chechi, Karthika chechi, Ashwathy chechi,Sivakka, Alby chechi, Lishma chechi*** *and* ***Sumbula chechi*** *for their constant support and motivation during the research programme. A special mention to thank all my dear juniors* ***Anjali, Sandra, Karolsha, Silpa, Geethika*** *and* ***Rengima*** *who helped me in one way or other.*

Word's can't express my soulful gratitude to my loving husband ***Mr. Nivin*** *for supporting me emotionally and making me strong every time without his sacrifice, boundless patience and unflagging interest this study would never have seen the light.*

I am in dearth of words to express my love towards my family especially ***my beloved parents, in-laws and brothers*** *for their boundless affection, moral support, eternal love, deep concern, prayers and personal sacrifice which sustains peace in life.*

I express my deep sense of gratitude to ***Kerala Agricultural University*** *for financial and technical support of my study and research work.*

It would be impossible to list out all those who have supported me in one or other way in the successful completion of this work. I once again express my heartfelt thanks to all those who helped me in completing this venture.

Anupama P.R

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Introduction

1. INTRODUCTION

Microorganisms associated with aerial parts of plants such as stems (caulosphere), leaves (phylloplane), flowers (anthosphere) and fruits (carposphere) which are collectively known as phyllosphere. The term 'phyllosphere' was coined by Last and Ruinen in 1955-56 (Levetin and Dorsey, 2006). Phyllosphere microbes include bacteria, filamentous fungi, yeasts and actinomycetes. They offer natural biological control of diseases affecting aerial plant parts. Being better adapted to the phyllosphere niche, they are potential biocontrol agents which are under-exploited in agriculture (Perazzolli *et al*., 2014). Hence, microbial biocontrol by phyllosphere microorganisms is a fast growing field of research.

Grain legumes are the second most important agricultural crops next to cereals, among which cowpea is important one adapted to tropics. Cowpea (*Vigna unguiculata* (L.) Walp.) is also known as food legume of 21st century. The probable reason for the name 'cowpea' is due to use of plant as hay for cows in Southeastern Americas and other parts of the world. In West Africa it is known as "niebe," "wake," or "ewa" and in Brazil it is known as "caupi" and in United States as "blackeyed peas", "southern peas", "field peas", "pink eyes" and "crowders" (Timko *et al*., 2007). It has different names such as "lobiya"(Hindi), "alasande"(Kannada), "payar"(Malayalam), "thattapayar"(Tamil) in India. Cowpea is a typical warm season crop believed to have originated in Africa (Padulosi and Ng, 1997).

Cowpea belongs to the family *Fabaceae* and it has two botanical varieties, the cultivated *Vigna unguiculata* var. *unguiculata* and the wild form *Vigna unguiculata* var. *spontanea*. Thelater is typically found mostly near the borders of cultivated cowpea fields. Cultivated cowpeas have been divided into five cultivar groups ("*unguiculata, sesquipedalis ,melanophthalmus*, *biflora, textilis")* based mainly on pod, seed and ovule characteristics. Out of five cultivar groups *"unguiculata"* is the largest which includes most of the African grain and forage types (more than 16 ovules/ pod). The *Vigna unguiculata* (L.) Walp. subspecies complex includes *aduensis, alba, baoulensis, burundiensis, dekindtiana, letouzeyi, pawekiae, pubescens, stenophylla, tenuis* and *unguiculata* (Pasquet, 1999).

In India, tender pods are used as vegetable and dry beans as pulse as high quality plant protein source. Cowpea is a major source of dietary proteins for the people of African countries and other developing countries of the world (Singh *et al.,* 2002; Langyintuo *et al.*, 2003). Hundred grams of green tender pods contains 4.3 g protein, 2.0 g fibre, 8.0 g carbohydrates, 74 mg phosphorus, 2.5 mg iron, 13.0 mg vitamin C, 80 mg of calcium, 74 g of phosphorous and 2.5 mg of iron *etc.* (Gopalakrishnan, 2007). Recently, cowpea gained more attention from consumers and researchers worldwide because of its exerted health benefits like anti-diabetic, anti-cancer, anti-hyperlipidemic, anti-inflammatory and antihypertensive properties (Jayathilake *et al.,* 2018).

Due to its nutritive value and soil improving properties, it is also used as a fodder, green manure and cover crop. In Kerala, it is grown as floor crop in coconut gardens, fringe crop in rice fields, intercrop with tapioca and in garden lands. The plant grows and covers the ground very swiftly and prevents soil erosion. It has the ability to tolerate drought and performs well in wide variety of soils. Being a leguminous crop, it replenishes nitrogen low fertility soils through nitrogen fixing bacteria. The crop demands low fertilizer application because its ability to fix atmospheric nitrogen around 80-90 kg per hectare under ideal condition. Since it restores soil fertility for succeeding crops like cereals, it is best suited for crop rotation (Carsky *et al*., 2002; Tarawali *et al*., 2002; Sanginga *et al*., 2003).

Cowpea is grown across the world on an estimated 14.5 million ha of land and the total annual production is 6.2 million metric tons. Africa ranks first with 5.2 million tonnes per hectare (95.2 per cent) followed by Asia with 1.5 million tonnes per hectare (2.9 per cent), America with 0.7 million tonnes per hectare (1.4 per cent) and Europe with 0.2 million hectare (0.5 per cent) (FAO, 2019). India is one of the major countries contributing cowpea production in the world. In India, area under cowpea cultivation is around 3.9 million hectares with 2.21 million tonnes production. It is also grown as a minor pulse crop cultivated mainly in arid and semi arid tracts of Punjab, Haryana, Delhi, and West Uttar Pradesh along with considerable area in Rajasthan, Karnataka, Kerala, Tamilnadu, Maharastra and Gujarat (Giridhar *et al*., 2020).

Cowpea production encounters a number of constraints, including pests and diseases from seedling to harvest that limit its production and yield (Adebanjo and Bankole, 2004; Akinbode and Ikotun, 2008). Major diseases include anthracnose caused by *Collectotrichum lindemuthianum,* dry root rot caused by *Macrophomina phaseolina*, leaf spot caused by *Cercospora cruenta,* rust caused by *Uromyces phaseoli* var. *vignae* and mosaic disease caused by *Cowpea mosaic virus.* Among the diseases, anthracnose caused by *Collectotrichum lindemuthianum* is a serious issue in all over country. It affects stem, vines, petioles, leaves and pods and results in substantial reduction in yield and seed quality. The disease management methods include application of fungicides, plant extracts, biocontrol agents and developing disease resistant varieties *etc*.

Cowpea phyllsophere possess a number of beneficial microbes which inhibits the pathogen causing anthracnose disease of cowpea (Adebanjo and Bankole, 2004). Hence, the present investigation was taken up to find out beneficial microbes present on cowpea phyllosphere to manage cowpea anthracnose. The study included the following experiments.

1. Purposive sampling survey to collect infected and healthy plant samples from farmers' field.
2. Isolation and characterization of the pathogen.
3. Isolation of phyllosphere microorganisms and *in vitro* screening for antagonism.
4. Characterization of phyllosphere antagonists.
5. *In vivo* evaluation of potential phyllosphere antagonists under rain shelter using randomized block design.
6. Studies on induced systemic resistance in cowpea by phyllosphere antagonists.

Review of Literature

2. REVIEW OF LITERATURE

Anthracnose is a serious disease of cowpea affecting mainly stem and spread to various parts of the plant. The disease is widely spread in India, China, Brazil, Ethiopia, Uganda, South Africa, Nigeria and various parts of Asia. In India, *Colletotrichum lindemuthianum* causing cowpea anthracnose disease has been reported by Butler in 1918, followed by Majid in 1950 (Assam), Mathur in 1954 (Uttar Pradesh) and several others. In association with anthracnose, brown blotch was also been observed in cowpea infected with *C. lindemuthianum* in Upper Volta and Zambia (Allen, 1983). Up to 50 per cent of yield loss has been reported in susceptible cultivars under humid wet conditions (Dada, 1990). The term "Anthracnose" was first used by Fabre and Dunal to describe a disease of grapes in which blackening of tissue was a characteristic feature and it literally means "like coal" usually sunken. It is caused by certain imperfect fungi that produce conidia in acervuli which are hyaline, one-celled, that is *Colletotrichum* (Jha *et al*., 2012). In India, the pathogen C. *lindemuthianum* infects all the aerial parts of the plant and productivity of the crop has been reduced upto 50 per cent (Satpathy and Beura, 2021).

The primary source of inoculum is seed and secondary sources are rain splash, air currents and contact with man and animals. Wet and humid conditions during growing season are favourable for anthracnose disease (Singh *et al*., 1997).The severity of the disease increased by a high plant population. It has been reported that the vertical and horizontal spread of disease is favoured by heavy and frequent rains with moderate temperature of 19-25°C and high relative humidity (more than 70 per cent) (Kumar, 1999). Infection of a susceptible cultivar under favourable conditions leads to epidemics which may result in 100 per cent yield loss (Fernandez *et al*., 2000). Bean anthracnose is a serious disease under cool and humid environments and the yield losses may be up to 100 per cent (Padder *et al*., 2017). Anthracnose caused by the fungus *C. lindemuthianum* is the most destructive disease of cowpea (Pradhan *et al.,* 2018). In Kerala, the climatic conditions are more favourable for the pathogen and disease is widely spread in all the districts.

2.1 Pathogen

The pathogen *Colletotrichum lindemuthianum* is believed to be the causal agent of anthracnose in legumes which is known to have races that vary from, country, region, location, and variety to another. Despite extensive pathological and molecular studies, the nature and extent of pathogen variability and its biology in *C. lindemuthianum* remain unknown. *C. lindemuthianum* is a hemibiotrophic fungus belongs to Melanconiaceae family, Melanconiales order, under class Deuteromycetes. Lindemuth reported the fungus in 1875 (Tiffany and Gilman, 1954). The name *C. lindemuthianum* is reported by mycobank. It is also known to cause brown blotch disease in cowpea and cause significant yield loss especially from pod infection (Allen, 1983).

The fungus was introduced to Pakistan from Nigeria through infected seeds and seed borne nature of the disease was reported for the first time (Emechebe and Mc Donald, 1979). It also survives on infected plant debris and losses often result from poor seed germination, reduced yield and decreased value of product (Allen, 1983). In Kerala, *C. lindemuthianum* is reported as the main pathogen causing anthracnose and also found that the disease is seed borne (Kumar, 1999). *C. lindemuthianum* race 73 causing dry bean anthracnose has been the first report from North Dakota in 2002 (Del Rio *et al*., 2002).

Apart from this, other species under the genus *Colletotrichum* has known to cause anthracnose in cowpea. *C. dematium* (Pers.) Grove is one of the most devastating species which cause substantial yield loss (Emechebe and Florini, 1997). Involvement of *C. gloeosporioides* f.sp. *aeschynomene* has been reported by Singh *et al.*, 1997. *C. destructivum* (O'Gara) is one of the major pathogen affecting cowpea production in the humid tropics (Adegbite and Amusa, 2008; Enyiukwu *et al*., 2014). First report on *Colletotrichum fructicola*as the causal agent of anthracnose in common bean and cowpea from Iran (Atghia *et al.*, 2015).Various species of *Colletotrichum* associated with the disease such as *C. gloeosporioides, C. fragerie, C. dermatium, C. lindemuthianum and C. destructivum* (Enyiukwu, 2017). Morphological variability studies of different isolates of *Colletotrichum* spp., conidial length and breadth of *Colletotrichum* spp. ranged between 15.95 - 27.00 x 2.50- 4.84 µm and setae size varied from 140.75 -249.21 x 4.31-5.96 µm (Tamin *et al*., 2020).

2.2 Symptomatology

The isolates of *C. lindemuthianum* from cowpea is distinct from beans in their external manifestation, which mainly cause stem disease in cowpea (Onesirosan and Barker, 1971). On infected pods, acervuli (fruiting bodies) resembling small pin cushions surrounded by setae are seen. Another prominent symptom is purplish brown discoloration of petioles, leaf veins, stems, peduncles and especially pods. Discoloration may be accompanied by cracking of stems. Symptoms first appear either at the stem base before flowering, or on pedicels (floral cushion) following flowering (Allen, 1983). Symptoms are brown that expand quickly and coalesce to girdle stems, peduncles and petioles on,susceptible species of cowpea (Allen, 1983; Smith *et al*., 1999). The disease manifest as tan to brown spots that appear sunken and form small circles on leaves, stems and branches. In severe cases, the flowers are also not spared, while the pods are covered with black spots which contain spores of the pathogen (Adegbite and Amusa, 2008). Anthracnose affects all the stages of the plant but more often in the reproductive stages. Its symptoms include round brownish or purple specks which become darker and enlarge into lesions (about 2 cm in diameter). The individual lesions are usually lenticular to circular, tan to brown in coloration and the size and distribution depend on the degree of severity (Sharon and Douglas, 2011). Disease is characterized by deep sunken black coloured lesion on stem which constrain its economic production (Enyiukwu and Awurum, 2013). The fungus infects all the aerial parts of the plant mostly through development of lesions. Such lesions create tan to brown irregular sunken spots which expand quickly and merge to stems, petioles and the entire plant (Satpathy and Beura, 2021).

2.3 Disease management

2.3.1 Host plant resistance

Cowpea genotypes differ in their susceptibility to the pathogen and use of resistant varieties controls anthracnose disease (Singh *et al*., 1997). Due to high variability and phenotypic plasticity, the fungus becomes more virulent and plants become susceptible in this climate change scenario. However there is a need for effective alternate methods to

save the crop from anthracnose disease. Field-type cowpeas show various levels of resistance, whereas pole-type vegetable cowpeas are highly susceptible (Kumar, 1999).

Round the world, based on utility, cowpeas are classified into field and vegetable types (Narayanankutty *et al.,* 2005).In contrast to this, a trailing-type vegetable cowpea (ssp. *sesquipedalis*), Arimbra Local from farmers's field of Malappuram district of Kerala has been reported to possess resistance to this disease (Shiny *et al*., 2015). Field-type cowpeas show various levels of resistance, whereas pole-type vegetable cowpeas are highly susceptible (Pradhan *et al*., 2018).

2.3.2 Chemical control

Chemical control is the most common approach adopted by growers to manage crop diseases. The control of anthracnose of cowpea has been sought through the use of fungicides like carbendazim, mancozeb, hexaconazole, SAAF *etc.* Use of foliar fungicides such as benomyl and carbendazim can reduce epidemics by 40 to 45%, but some strains of *Colletotrichum* species with resistance to fungicides such as carbendazim and thiophanate-methyl have been discovered in India (Emechebe and Florini, 1997). Mancozeb found to be the best fungicide against cowpea anthracnose concerned with crop yield and b:c ratio (Kumar, 1999).The minimum disease intensity and disease incidence was recorded in treatments (Carbendazim 12 per cent + mancozeb 63 per cent) SAAF at 0.2 per cent (Ahmad *et al*., 2018).

Integrated management package which includes seed treatment with carbendazim (2 g/kg seed), soil solarization for a period of 45 days, application of *Trichoderma* enriched neem cake organic manure mixture @ 1 kg/pit 15 days after seed emergence, application of tebuconazole (0.1 per cent) at 30, 45 and 60 days after seed emergence (Sreeja *et al*., 2015).

2.3.3 Biological methods

Due to the increased awareness on the side effects of chemical fungicides, increased cost, toxic residue in soil and environment and development of resistance, much attention

is being given on the alternative methods of pathogen control like the use of eco- friendly methods such as bio control agents or plant extracts.

2.3.3.1 Control by botanical extracts

Alcohol and water extracts of *Piper nigrum*, *Ocimum sanctum* L. and *Citrus limon* (L.) are considered to be effective in reducing diseases of *Colletotrichum* spp. of cowpea *in vitro* and *in vivo* conditions (Amadioha, 2003). Garlic crude extract at 45 per cent concentration showed 100 per cent inhibition of *C. lindemuthianum in vitro* (Ajayi and Oydele, 2016). Hot water extracts of *Ricinus communis*, *Jatropha gossypifolia* and *Datura stramonium* at three concentrations (65, 50 and 30 per cent) compared with benomyl shows that extracts of these indigenous plants can be used as a substitute for the benomyl fungicide and that they are more effective when used as a preventive method in the management of anthracnose disease (Falade *et al.,* 2017). Foliar spray of extracts of *Azadirachta indica*, *Acalypha wilkisiana* and *Carica papaya* at four stages such as three weeks after planting, flowering stage, at the initial podding stage and at the full podding stage reduced incidence and severity of anthracnose caused by *C. lindemuthianum* which resulted in yield increase (Ganiyu *et al.,* 2018). Seed treatments with Ajwain seed extract + spray of Ajwain seed extract was found effective in reducing disease incidence (42.97 per cent) and increase in grain yield by 54.86 per cent (Ahmad *et al*., 2018). Application of neem followed by ginger, onion, garlic and tulsi extracts effectively reduce the percent disease incidence ranging 70- 90 per cent and *Lantana camara* extract at 50 – 100 per cent gave the best disease control and yield improvement of the crop (Enyiukwu *et al.,* 2021). Similarly, the percent green pod yield over control as well as the cost-benefit ratio which can be recommended to the farming community (Satpathy and Beura, 2021).

2.3.3.2 Biological control by microbial agents

Sustainability in agriculture can be achieved by incorporation of biocontrol agents along with cultural practices. It is an eco friendly and effective method in which beneficial microbes are used for improving plant health and to induce resistance against harmful pathogens.*T. viride* has the maximum potentiality to suppress the spore germination, mycelial growth, seed borne infection of *C. lindemuthianum* and increased seed

germination (Padder and Sharma, 2011). *T. asperellem* showed lowest values for disease incidence caused by *C. lindemuthianum* and the highest value for pod yield (Ajayi and Oydele, 2016). Application of *Trichoderma* sp. as seed treatment + foliar spray were also effective in reduce disease incidence (39 per cent) and increase grain yield (50 per cent) (Ahmad *et al*., 2018).

2.3.3.2.1 Rhizoshere microorganisms

Rhizosphere is the narrow region of soil or substrate that is directly influenced by root secretions and associated soil microorganisms. The rhizosphere microbiome is critical for plant growth and protection against plant pathogens which includes fungi, bacteria, yeast, actinomycetes *etc. B. amyloliquefaciens* isolated from the suppressive soil used against panama disease caused by *Fusarium oxysporum* f. sp. *cubense*, resulted in reduced disease incidence by 68.5 per cent and doubled the yield (Xue *et al*., 2015). *T. harzianum* strain CCTCC-RW0024 from rhizosphere is a potential biocontrol agent against fusarium stalk rot caused by *Fusarium graminearum* (Saravanakumar *et al*., 2017). *Pseudomonas putida* strain AKP -1 isolated from rhizosphere of sugarcane inhibited the pathogen *C. falcatum* (69.2 per cent) by producing catalase, protease and HCN also enhanced sugarcane seedling growth under green house condition (Verma *et al.*, 2018).

2.3.3.2.2 Endophytes

Bacterial endophytes isolated from roots of plants under fabaceae family such as *Bacillus subtilis* subsp. *subtilis*, *Bacillus atrophaeus*, *B. tequilensis*, *B. subtilis* subsp. *spizizenii*, *Streptomycescyaneofuscatus*, *S. flavofuscus*, *S. parvus*, *S. acrimycini* were showed promising inhibition of *C. lindemuthianum* (Gholami *et al*., 2013).

2.3.3.2.3 Phyllosphere microorganisms

Phyllosphere refers to the total above plant surface of the plant which is further divided into caulosphere (stems), phylloplane (leaves), anthosphere (flowers) and carposphere (fruits) which is characterized by a variety of microorganisms including bacteria, filamentous fungi and yeast as pathogens, saprobes and epiphytes in various plant species (Levetin and Dorsey, 2006). The phyllopshere microflora include organisms such

as bacteria, filamentous fungi, yeasts, algae and protozoans and structure of phyllosphere communities reflects immigration, survival and growth of microbial colonists, which is influenced by numerous environmental factors in addition to leaf physico-chemical properties (Whipps *et al*., 2008). The phyllosphere is colonized by complex microbial communities, which are adapted to the harsh habitat. Although the role and ecology of nonpathogenic microorganisms of the phyllosphere are only partially understood, leaf microbiota could have a beneficial role in plant growth and health (Perazzolli *et al.,* 2014).

The above-ground surfaces of plants (phyllosphere) harbour a diverse variety of microorganisms, and this phyllosphere microbiome interacts with the host plant affecting its health and function. Phyllosphere microorganisms, predominantly bacteria and fungi, can act as mutualists promoting plant growth and tolerance of environmental stressors, commensals using the leaf habitat for their own growth and reproduction, or as antagonistic pathogens (Stone *et al*., 2018). The plant leaf surface, or phyllosphere, represents a unique and challenging microbial biome with a diverse and dynamic community of commensal, parasitic and mutualistic agents of microscopic proportions (Leveau, 2019). A fast-growing field of research focuses on microbial biocontrol in the phyllosphere. Phyllosphere microorganisms possess a wide range of adaptation and biocontrol factors, which allow them to adapt to the phyllosphere environment and inhibit the growth of microbial pathogens, thus sustaining plant health (Legein *et al*., 2020). Phyllosphere microbiome enhance plant tolerance to withstand biotic and abiotic stress conditions in the current facet of climate change (Pandiyan *et al*., 2021). From these results it is evident that the microbes inhabiting phyllosphere provide natural protection for plants against major diseases.

2.4 Factors influencing population of phyllosphere microflora

The population of microbial communities inhabiting phyllosphere altered by many factors such as temperature, humidity, UV radiations, fungicides, pesticides and other chemicals.At higher temperature greater amount of colonization and stability of *B. amyloliquefaciens isolate* CC09 was seen in wheat phyllosphere (Hongfeng *et al*., 2014).Apple phyllosphere microbiota were affected by UV radiations(Glenn *et al*., 2015). Phyllosphere microbiota differ significantly between plants grown in open field and under protection (Wei *et al*., 2016). Beneficial microbial communities present in phyllosphere

also get affected by chemical management methods which result in negative consequences for plant health and productivity. It was found that commonly used fungicides had moderate but significant effect on fungal community composition in the wheat phyllosphere. The relative abundance of several saprotrophs was altered by fungicide use (Karlsson *et al*., 2017). Metagenomic analysis revealed that foliar application of combination of iprodione + carbendazim (systemic fungicide) for the management of early blight of tomato, affectes more on non-target leaf fungal communities than contact fungicide propineb (Sumbula *et al*., 2022).

2.5 Enhanced growth and vigour of plants by phyllosphere microorganisms

The plant growth and protection against pathogens are influenced by physiological activities, of the phylloplane colonizers. *Trichoderma longibrachiatum* can be considered to be a promising bio-control agent against *Meloidogyne incognita* infecting cucumber with a high efficacy and, increased plant height, root length, shoot and root fresh weight (Zhang *et al*., 2015). When plants were pre- treated with *B. amyloliquefaciens* as bicontrol for chilli anthracnose under greenhouse conditions resulted in maximum enhancement of seed germination (84.75 per cent), seedling vigor index (1423.8) along with an increase in vegetative growth parameters and significant disease protection of 71 per cent was observed (Gowtham *et al*., 2018). *T. asperellum* TC01 is effective against infected with *C. gloeosporioides* C62 infecting tea plants and showed plant growth promotion (Shang *et al*., 2020).

2.6 Phylloshere fungi

Filamentous fungi are more abundant in plant phyllosphere ranging from 10^2 to 10^8 CFU g^{-1} which includes certain genera such as *Cladosporium, Alternaria, Pencillium, Acremonium, Mucor* and *Aspergillus* colonizing as epiphytes and endophytes (Arnold *et al*., 2001). Fungal isolates from cowpea phylloplane such as *Aspergillus flavus*, *A. niger, A. ochraceus, Penicillium aurantiogriseum, T. viride* -TH14, *T. viride*-TH31 inhibited *in vitro* and *in vivo* growth of *C. lindemuthianum* causal agent of cowpea anthracnose disease (Adebanjo and Bankole, 2004).

2.7 Phyllosphere bacteria

Global scale estimation gives the phyllosphere spans more than 10^8 km^2 and is habitat upto 10^{26} bacterial cells (Lindow and Brandl, 2003). The aerial surface of plants is ubiquitous global habitat that harbours diverse bacterial communities. They were dominated by a core microbiome of taxa including actinobacteria, alpha, beta, gamma-proteobacteria and sphingobacteria (Vorholt, 2012). Many of the dominant taxa in the phyllopshere belong to clades known to associate closely to plant as diazotrophic and methylotrophic (Phillippot *et al*., 2010) The members of the orders Bacillales and Pseudomonadales were found as strong inhibitors of plant pathogens (Blin *et al*., 2019).

Bacterial isolates obtained from cowpea phylloplane *viz. Bacillus subtilis*- BS21, 22 and 23 inhibited *C. Lindemuthianum* under *in vitro* and *in vivo* conditions (Adebanjo and Bankole, 2004). *In vitro* analysis of rice phyllosphere bacteria *Bacillus subtilis* subsp. *subtilis* is effective against *Pyricularia oryzae* race 173 which reduced blast incidence upto 70.83 per cent by producing antifungal compounds (Wiraswati *et al*., 2020).

2.8 Phyllosphere yeasts

Yeast densities in phyllosphere of different field grown plants ranged between 1.4x10 to 4.3 x 10^3 cells/g. Highest population was seen in grape and wheat phyllosphere and least population count was reported from cabbage and cowpea. It includes *Rhodotorula glutinis*, *Cryptococcus albidus, C. diffluens*, *Torulopsis famata*, *T aeria*, *Candida curvata* , *C. humicola* and *Debaromyces kloeckrii.* Spore forming yeasts were less dominant in the phyllosphere (El Din *et al*., 1986). *Candida tropicalis* isolated from phyllosphere inhibited the mycelial growth of the pathogen *C. gloeosporioides* (Sriram and Poornachanddra, 2013) and *C. tropicalis* isolate YZ1 and YZ27 significantly reduced anthracnose disease severity of harvested banana caused by *C. musae* by competing for space and nutrients and rapid colonization of the yeast was seen when applied on wounds (Zhimo *et al*., 2016). *Candida tropicalis* VYW1 obtained from mungbean nodules significantly influenced nodulation behavior, plant growth and soil health by exhibiting increased nodules per plant, nodule biomass (Annadurai *et al*., 2020).

2.9 Phyllosphere actinomycetes

Actinobacteria share a considerable interest in epiphytic and endophytic life forms in the phyllosphere. In tropical and temperate ecosystem, the diversity of phyllopsphere actinomycete was found more (Strobel and Daisy, 2003). Several species of actinobacteria were reported from plants which includes *Nocardiodes* sp., *Pseudonocardia*, *Streptomycesetc*. (Yadav and Yadav, 2019).

2.10 INDUCED SYSTEMIC RESISTANCE AGAINST ANTHRACNSOE DISEASE

Plants possess a range of active defence apparatus that can be actively expressed in response to biotic stresses such as diseases and pests. Induced resistance is a state of enhanced defensive capacity developed by a plant when stimulated by external or internal sources. There are two forms of induced resistance systemic acquired resistance (SAR) in which plant defences are preconditioned by prior infection by pathogens and induced systemic resistance (ISR) is stimulated by beneficial microorganisms. The systemic resistance in plants is mainly mediated by defence related genes that encode a variety of proteins such as enzymes controlling secondary metabolism, pathogenesis related proteins (PR) and regulatory proteins (Dixon *et al*., 1994). The major enzymes involved in systemic resistance in plants are peroxidase, polyphenol oxidase catalyzes the formation of lignin and phenylalanine ammonia lyase that is involved in production of phytoalexins and phenolics.

Bacteria such as *Bacillus* spp. and *Pseudomonas* spp. elicit plant growth promotion and induce systemic resistance.They can activate ISR which is independent of salicylic acid but dependent on jasmonic acid, ethylene and regulatory gene *NPR 1* (Kloepper *et al*., 2004). Combination of ISR and SAR can increase protection against pathogens that are resisted through both salicylic acid (SA) and jasmonic acid (JA) pathways besides extended protection to a broad spectrum of pathogens than ISR/SAR alone. Plant growth promoting rhizobacteria (PGPR) play a major role in disease suppression by antagonism as well as inducing systemic resistance against plant pathogens. Several specific strains of species *B. amyloliquifaciens*, *B. subtilis, B. pasteurii, B.cereus, B. pumilus, B. mycoides*, and *B. sphaericus* elicit significant reduction in the incidence or severity of various diseases on a

diversity of hosts (Choudhary *et al*., 2007). Rhizobacterial treatments for the management of bacterial wilt of ginger revealed higher activity of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase after challenge inoculation (Vijayaraghavan and Abraham, 2011). *Bacillus cereus* AR156 is an important growth promoting rhizobacterium which induce resistance in plants against a broad spectrum of pathogens including *Pseudomonas syringae* pv. *Tomato* DC3000 by producing defence related genes *PR1,PR2,PR5* and *PDF1.2* and simultaneous activation of salycilic acid (SA), jasmonic acid (JA) and ethylene (ET) dependent signaling pathways when treated and lead to additive effect on the level of induced protection (Niu *et al*., 2011).

Higher activity of peroxidase and polyphenol oxidase were reported in cocoa plants treated with promising endophytes against *Phytophthora palmivora* (Kurian, 2011). The application of biocontrol agents such as *Trichoderma viridae*, *Pseudomonas fluorescens*, *Bacillus subtilis* in anthurium plants against *Colletotrichum gloeosporiodes* revealed that these bicontrol agents triggered the activity of defence related enzymes such as peroxidase, polyphenol oxidase and phenylalanine ammonia lyase (Selvaraj and Ambalavanan, 2013).

Plant growth-promoting rhizobacteria enhance the immunity of above-ground tissues, which is known as induced systemic resistance (ISR). Some microbes are able to activate plant defence mechanisms, including systemic acquired resistance (SAR) and induced systemic resistance (ISR). While SAR is induced in systemic tissues of plants undergoing a local pathogen infection, ISR takes effect in aerial tissues of plants interacting with beneficial microbes in the rhizosphere (Vlot *et al*., 2021).

Hence in this light of available literature, this study mainly aimed for finding out phyllosphere microflora inhabiting cowpea, their role in host resistance against anthracnose causing pathogen and characterization.

Materials and Methods

3. MATERIALS AND METHODS

The project entitled "Phyllosphere microorganisms for the management of anthracnose disease of cowpea (*Vigna unguiculata* (L.) Walp.)" was carried out in the Department of Plant Pathology, College of Agriculture, Kerala Agricultural University, Vellanikkara during 2019-2021. The details of materials used and methodologies followed during the course of study are described in this chapter.

3.1 COLLECTION, ISOLATION AND IDENTIFICATION OF THE PATHOGEN ASSOCIATED WITH COWPEA ANTHRACNOSE DISEASE

Leaf, stem and pods of cowpea, showing anthracnose disease symptoms were collected from different locations of Kerala (Thrissur, Palakkad, Malappuram, Kasaragod districts) (Plate 3.1& 3.2). The samples were washed under tap water and small bits were cut from infected areas along with healthy portion. These bits were surface sterilized using one per cent sodium hypochlorite solution for one minute, followed by washing in changes of sterile water. The surface sterilized bits were placed on Petri plates containing potato dextrose agar (PDA) and incubated at 28+/-2ºC. When mycelial growth was visible, small bits of the growth were transferred to fresh PDA slants (Rangaswamy, 1958).The cultural and morphological characters of the isolates *viz.* growth rate and pattern of growth, width of hyphae, size of conidia were studied.

Pathogenicity of the isolates was proved by artificial inoculation on healthy cowpea plants. Mycelial disc of (10mm) of the isolate from seven day old culture grown on potato dextrose agar was taken using cork borer and placed on pin pricks made on stem and leaves of healthy plant. Cotton moistened with sterile water was placed over the mycelial disc. The inoculated plants were kept in moist chamber. Observations were recorded. The organisms were re-isolated from pods which were infected by artificial inoculation (Plate 3.3). The cultural and morphological characters of the pathogen isolates were studied. The isolates were purified by hyphal tip method (Rex *et al.,* 2019) and maintained on PDA slants for further studies. The isolates were catalogued based on the name of location from which the samples are collected.

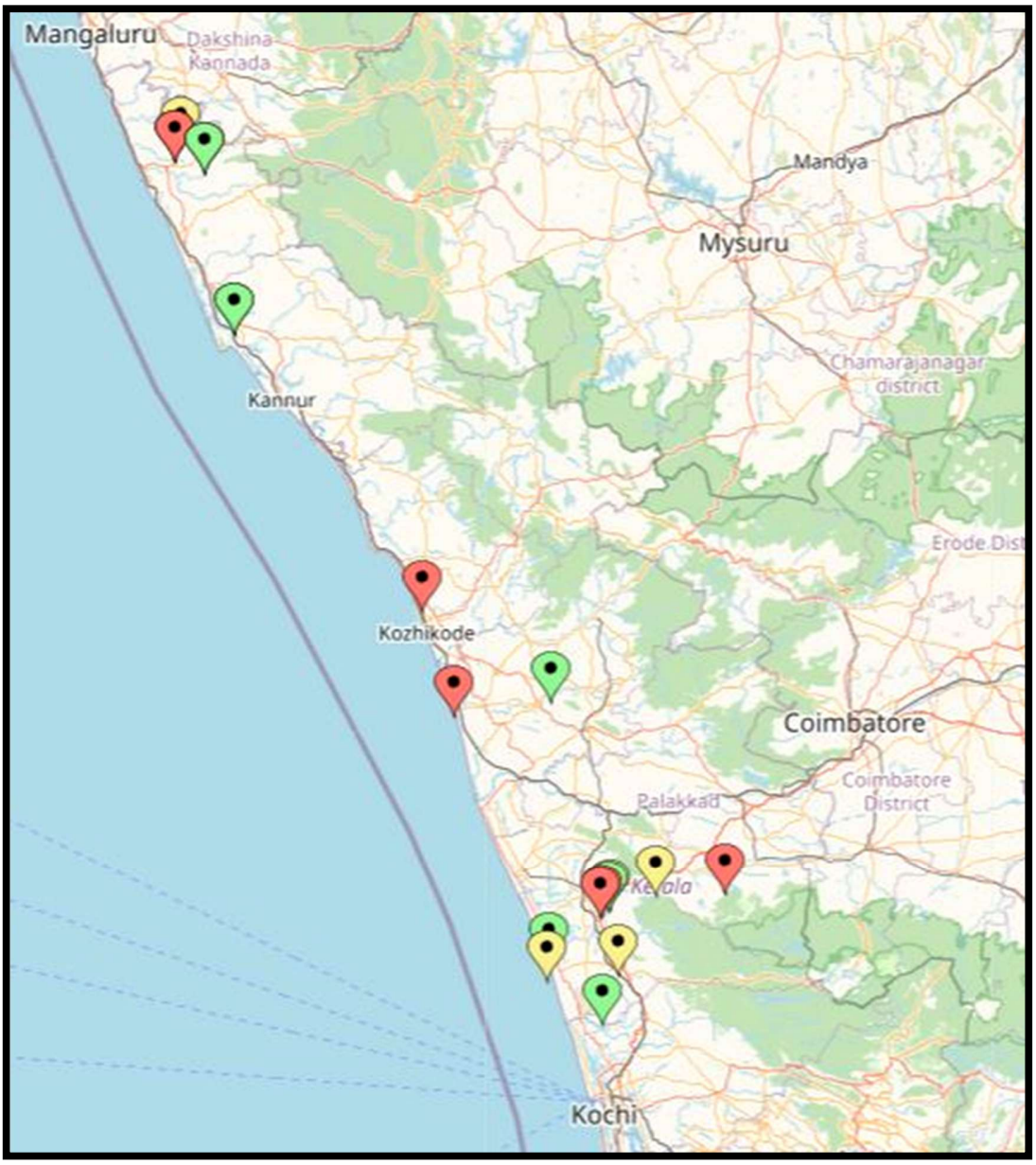
Table 3.1 Locations surveyed

District	Location	AEU	Latitude/ Longitude	Temp. (°C)	RH(%)
Thrissur	Kallingalpadam	NCL(AEU 10)	10.6824°N /76.4230°E	26.2	79
	Madannur	NCL (AEU 10)	10.6824°N /76.4230 °E	26.4	79
	Pandallur	NCL (AEU15)	11.4884°N /76.3391 °E	28.3	59
	Mala	KL (AEU06)	10.2403°N /76.2631°E	28.2	59
	Kazhimbram	KL (AEU06)	10.3997°N /76.1160 °E	28.9	78
	Natika	KL (AEU06)	10.4275°N /76.0943 °E	28.9	78
	Vidya Nagar	NCL (AEU 10)	10.5452°N /76.274°E	27	88
	Bosco Nagar	NCL (AEU 10)	10.5452°N /76.274°E	28	70
	Vellanikkara	NCL (AEU 10)	10.5452°N /76.274°E	26	86
	Madakkathara	NCL (AEU 10)	10.5452°N /76.274°E	29	82
	Chirakkekode	NCL (AEU 10)	10.5578°N /76.2905°E	25.9	86
	Mudicode	NCL (AEU 10)	10.5578°N /76.2905°E	28.8	68
	Ollukkara	NCL (AEU 10)	10.5319°N /76.2523°E	27	88
	Mannuthy	NCL (AEU 10)	10.5452°N /76.274°E	29	70
Palakkad	Nenmara	NFH (AEU13)	10.5934°N /76.6006 °E	28.2	62
Malappuram	Manjeri	SHH (AEU15)	11.1203°N /76.1199°E	26.4	68
	Ariyallur	NCP (AEU02)	11.0927°N /75.8447 °E	27.8	74
Kozhikode	Thalakulathur	NL (AEU11)	11.3496°N /75.7597 °E	26.4	78
Kannur	Payyannur	NL (AEU11)	12.0972°N /75.1934 °E	27.2	58
Kasaragod	Mulleria	NL (AEU11)	12.5510°N /75.1633 °E	26.2	70
	Ukkinadka	NL (AEU11)	12.6426°N /75.1049 °E	27	72

NCL - Northern central laterite, NL -Northern laterites, NCP- Northern coastal plains,

SHH - Southern high hills, KL – Kole land,NFH –Northern foothills

Plate 3.1 Map showing different locations of sampling survey



Infected samples collected

Healthy samples collected

Both healthy and infected samples collected

Table 3.2: Standard score chart for assessing cowpea anthracnose disease

Sl no.	Score	Symptoms
1	0	Healthy plants with no visible symptoms
2	1	Few anthracnose lesions on main stems and petioles of lower leaves only
3	2	Anthracnose lesions on stems, petioles and branches only
4	3	Moderate anthracnose lesions on stems, petioles, branches and veins on the abaxial leaf surfaces
5	4	Severe anthracnose lesions on stems, petioles, branches, leaf veins and peduncles
6	5	Very severe infection on all parts of the plant including the pods

3.1.1 Disease assessment

The per cent disease incidence (PDI) in different fields was calculated using the formula given by Wheeler (1969) as given below:

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

3.1.2 Assessment of disease severity

The severity of anthracnose was measured using a 0-5 scale score chart developed by Dada, 1990 (Table 3.2).

3.2 CHARACTERIZATION OF THE PATHOGEN

The identification of pathogens causing anthracnose disease in cowpea was done based on the cultural, morphological and molecular characterization.

3.2.1 Cultural characterization

Cultural characterization of pathogens isolated from infected samples were sub cultured on sterilized and solidified potato dextrose agar (PDA) and kept for incubation at room temperature (26 + 2°C). Visual observations like growth rate of the fungus, colour of mycelia, pattern of growth, texture of mycelia, pigmentation, sporulation, presence of fruiting bodies, production of different fluids/ooze in the culture, colour on the reverse side of the Petri plate were noted.

3.2.2 Morphological characterization

The morphological characters of the fungus which include hyphal colour, hyphal branching pattern, presence of septation, presence of conidia, septation on conidia, colour, shape and size of spores, dimensions of spores, type and size of fruiting bodies were recorded with the help of microscope.

3.2.3 Molecular characterization

The molecular characterization was carried out at Rajiv Gandhi Centre for Biotechnology, Thirvuvananthapuram and the fungus was identified at species level.

3.2.3.1 Isolation of genomic DNA of fungus

The fungal DNA isolation was done using NucleoSpin® Plant II Kit (Macherey-Nagel)

- 100mg of the tissue/mycelium is homogenized using liquid nitrogen and the powdered tissue is transferred to a microcentrifuge tube.
- 400µl of buffer PL1 is added and vortexed for 1 minute and 10µl of RNase A solution is added and inverted to mix.
- The homogenate is incubated at 65°C for 10 minutes and the lysate is transferred to a Nucleospin filter and centrifuged at 11000 x g for 2 minutes.

Plate 3.2 Anthracnose symptoms on cowpea

Stem girdling

Abaxial surface of leaf

Acervuli with setae as pin cushions

Plate 3.3 Pathogenicity test

Pathogen isolate

Inoculated on leaves inside moist chamber

Reisolation from the symptom

Symptom expression

- The flow through liquid is collected and the filter is discarded and 450 µl of buffer PC is added and mixed well.
- The solution is transferred to a Nucleospin Plant II column, centrifuged for 1 minute and the flow through liquid is discarded and 450 µl buffer PW1 is added to the column, centrifuged at 11000 x g for 1 minute and flow though liquid is discarded.
- Then 700 µl PW2 is added and centrifuged at 11000 x g. The flow through liquid is discarded and finally 200 µl of PW2 is added and again centrifuged at 11000 x g for 2 minutes to dry the silica membrane.
- The column is transferred to a new 1.7 ml tube and 50 µl of buffer PE is added and incubated at 65^{o}C for 5 minutes and the column is then centrifuged at 11000 x g for 1 minute to elute the DNA. The eluted DNA was stored at 4^{o}C.

3.2.3.2 Agarose Gel Electrophoresis

The quality of the DNA isolated was checked using agarose gel electrophoresis.One microlitres of 6X gel-loading buffer (0.25 per cent bromophenol blue, 30 per cent sucrose in TE buffer pH-8.0) was added to five microlitres of DNA.The samples were loaded to 0.8 per cent agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml 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).

3.2.3.3 PCR Analysis

The amplification of fungal ITS region of isolated DNA was carried out in a PCR thermal fcycler (GeneAmp PCR System 9700, Applied Biosystems). Reaction mixture contained 5 µl of 2X Phire Master Mix, 0.25 µl of forward primer, 0.25 µl of reverse primer, 1 µl of purified fungal template DNA and 4 µl of distilled water. The details of primers used for PCR amplification are given in the (Table 3.3). The amplification process was carried out with an initial denaturation at 98^{o}C for 30 sec, followed by 40 cycles of denaturation at 98^{o}C for 5 sec, annealing at 58^{o}C for 10 sec and extension at 72^{o}C for 15

sec. After the completion of 40 cycles of amplification, a final extension step was performed at 72^{o}C for 60 sec.

Table 3.3 ITS primers used for amplification

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

3.2.3.4 Gel documentation and ITS- rDNA gene sequencing

The PCR products obtained after the completion of reactions were run in 1.2 per cent agarose gel prepared with 0.5X TBE buffer containing 0.5 µg per ml of ethidium bromide to visualise the DNA banding pattern. 4 µl of PCR product mixed with 1 µl of 6X gel loading dye was loaded to the wells and the process was performed using 0.5X TBE buffer as running buffer. 2-log DNA ladder (NEB) was taken as the ladder to compare and identify the corresponding size of the DNA bands. When the dye moved one third of the gel, electrophoresis was done and the gels were visualized in a UV transilluminator (Genei) and the images of visualised DNA bands were captured using Bio- Rad Gel documentation system. Sequencing reaction was done in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems) using the BigDye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The components in the PCR reaction mixture are shown in the Table 3.3 and the steps in the PCR amplification for sequencing is given in the Table 3.4.

Table 3.4: Components of PCR mix for ITS sequencing reaction

Sl no.	Component	Quantity
1.	5X Sequencing Buffer	1.9 µL
2.	Forward Primer	0.3 µL
3.	Reverse Primer	0.3µL
4.	Sequencing Mix	0.2 µL
5.	ExoSAP treated PCR product	1.0 µL
6	Distilled water	6.6µL

Table 3.5: PCR amplification programme of ITS sequencing

Steps	Temperature	Duration	
Initial denaturation	96°C	2 min	
Denaturation	96°C	30 sec	} 30 cycles
Annealing	50°C	40 sec	
Extension	60°C	4 min	
Final extension	4°C	∞	

3.2.3.5 *In silico* analysis of ITS sequences

The sequences were analysed using BLASTn search against NCBI nr database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

3.3 ISOLATION AND ENUMERATION OF PHYLLOSPHERE MICROFLORA

Samples were collected from different locations of Thrissur district and other parts of Kerala. Freshly collected samples were brought separately in poly ethene covers and stored in refrigerator were used for isolation within 48 hours. Phyllosphere microbes were isolated from leaves, stems, flowers and pods of healthy plants using the dilution pour-plating technique as described by Bankole (1990). The area of the samples were measured using graphical method (Pandey & Singh, 2011) (Plate 3.4) and were cut into pieces and put in 100ml sterile water and shaken for 30 minutes. These washings were pour plated after serial dilution in 10^{-2}, 10^{-4} (Martin's rose Bengal agar for fungi and yeast), 10^{-4}, 10^{-5} (Kenknights's agar for actinomycetes), 10^{-6} and 10^{-8} (King's B, Nutrient agar for bacteria) respectively (Plate 3.5) and incubated at room temperature. Promising colonies were selected and cultured. Details of dilution and media used for isolation and enumeration of phyllosphere microorganisms are given in the Table 3.6.

Table 3.6 Media used and dilution of plant washings for the isolation of different phyllsophere microorganisms

Sl no.	Organism	Dilution	Medium	Period of incubation
1	Fungi	10^{-4}	Martin's rosebengal streptomycin agar	48h.
2	Yeast	10^{-4}	Glucose yeast extract Peptone Agar	48h.
3	Actinomycetes	10^{-5}	Ken Knight's Agar	Seven days
4	Bacteria	10^{-6}& 10^{-8}	Nutrient Agar	24h.
5	Fluorescent pseudomonads	10^{-6}& 10^{-8}	King's B Agar	24h.

Representative colonies of phyllosphere microbes based on colony morphology were selected and picked from the dilution plates and purified using standard protocols. Altogether 183 phyllosphere isolates were thus subcultured and their details such as location and part of the plant from which it was isolated and medium of isolation were recorded.

3.4*IN VITRO* ANTAGONISTIC EFFECTS OF PHYLLOSPHERE MICROBES AGAINST THE PATHOGEN

The *in vitro* antagonistic effect of phyllosphere microbes towards the pathogen was tested by dual culture method. Initially, 183 phyllosphere isolates were obtained from the experiment 3.2 were subjected to preliminary screening to test their interaction with the pathogen.

3.4.1. Preliminary screening of phyllosphere microbes against the pathogen

Mycelial disc of the pathogen taken from a seven day old culture on PDA was placed in the center of fresh PDA plate and incubated for 48h at 28+/- 2^{o}C. Phyllosphere bacteria and yeasts were inoculated as a line of streak on both sides of the culture disc of

Plate 3.4 Graphical method to measure the surface area of the samples

Leaf Stem Flower Pod

Plate 3.5 Preparation of plant washings for the isolation phyllosphere microorganisms

Plant samples soaked in sterile water (100 ml)

Shaking for 30 minutes

the pathogen, 2 cm away from the edge of the dish. The isolates which showed antagonism were selected and transferred to fresh medium. In the case of phyllosphere fungi, mycelial disc of isolates were placed one side of the Petri dish and another side with pathogen 2 cm away from the edge of the Petri dish. Plates with pathogen alone served as control. The plates were incubated at room temperature and observed for inhibition of the pathogen for five days or when there was full growth in the control. 75 isolates showed antagonism towards the pathogen during preliminary screening.

3.4.2 *In vitro* evaluation of antagonistic phyllosphere isolates

Out of 75 isolates only 41 phyllosphere isolates including 15 isolates of bacteria, 25 isolates of fungi and one yeast isolate which showed more than 60 per cent antagonism were selected and transferred to fresh medium and pure cultures were established. These isolates were sub-cultured at fortnightly intervals and maintained on test tube slants with nutrient agar for bacteria and Potato dextrose agar for fungi and yeasts. Bacterial isolates were stored in sterile water at 4°C. The antagonistic phyllosphere isolates selected based on the preliminary screening were further tested individually. The objective of this experiment was to select more efficient antagonists among the 41 isolates which showed antagonistic action in the preliminary screening (Plate 3.6).

3.4.2.1 *In vitro* evaluation of selected bacterial and yeast isolates

All the 25 isolates of bacteria and one yeast isolate were evaluated for their antagonistic effect by dual culture method (Utkhede and Rahe, 1983) (Plate 3.7A). Mycelial discs (8mm) taken from seven day old culture of the pathogen grown on PDA was placed at the centre of the mediated (PDA) Petri dish and bacterial/ yeast isolate were inoculated as a line of streak on both side, 2 cm away from the edge of the Petri dish and incubated at room temperature for seven days. Three replications were maintained for each isolate. Plates with the pathogen alone served as Control. The plates were incubated at room temperature and growth of the pathogen was observed daily, until the control exhibited full growth. The per cent inhibition of the pathogen was calculated using the formula suggested by Vincent (1927).

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

PI = per cent inhibition

C = growth of pathogen in control (mm)

T = growth of pathogen in dual culture (mm)

3.4.2.2 *In vitro* evaluation of antagonistic fungi

Twenty five fungal isolates were evaluated for their antagonistic action against the pathogen by dual culture method (Skidmore and Dickinson, 1976) (Plate 3.7B). The organisms were inoculated on dual cultures after giving due consideration on their growth rate. Mycelial disc (8mm) of the pathogen from seven day old culture grown on PDA was placed on one side of the plate and mycelial disc (8mm) of antagonistic fungi were placed on the other side if the plate, four centimeter away from the pathogen inoculated and incubated at room temperature for seven days.Three replication were maintained for each isolate. The pathogen grown on monoculture served as control. The plates were observed daily after 24h of inoculation of antagonist till the pathogen grew and covered the plate in control. The per cent inhibition was calculated as in the case of 3.3.2.1.

3.4.3 Selection of phyllsophere antagonists

Based on the dual culture screening, five isolates which showed more than 70 per cent inhibition of the pathogen were selected and code is given based on name of location and type of organism. They are used for testing their efficacy in early growth promotion and disease management under *in vivo* conditions.

CKDSF1= Chirakkekode (CKD), Stem (S), Fungi (F)

KVKSF2= Krishi Vigyan Kendra, Thrissur (KVK), Stem (S), Fungi (F)

KPCSB1= Kallingalpadam (KP), Cowpea Stem (CS), Bacteria (B)

MUCSB1= Mulleria (MU), Cowpea Stem (CS), Bacteria (B)

NKCSY1= Natika (NK), Cowpea Stem (CS), Yeast (Y)

Plate 3.6 Preliminary screening

Control

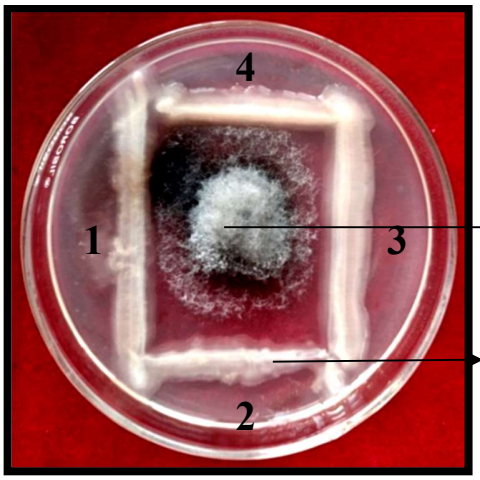
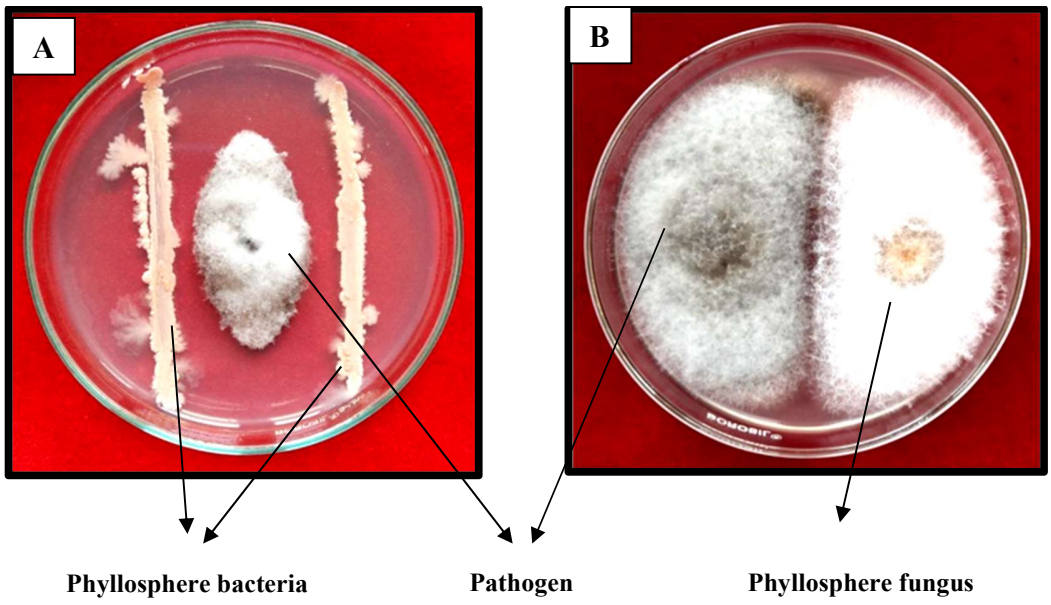


Plate 3.7 Secondary/dual culture screening of phyllosphere bacteria and fungus



3.5 CULTURAL AND MORPHOLOGICAL CHARACTERIZATION OF ANTAGONISTIC PHYLLOSPHERE MICROBES

3.5.1 Cultural and morphological characterization of antagonistic phyllosphere fungi

Same as explained in 3.2.1 and 3.2.2

3.5.2 Cultural and morphological characterization of antagonistic phyllosphere bacteria

Characterization of different promising bacterial isolates KPCSB1andMUCSB1 was carried out following methods suggested in the Manual of Microbiological Methods, published by the Society of American Bacteriologists (1957) and also by the Bergy's manual of systematic Bacteriology, Vol.1 (Stanely *et al*., 1989). The cultural and morphological characters of the isolates such as colony characters, Gram's reaction were studied.

3.5.2.1 Cultural characterization

Promising phyllosphere bacterial isolates were streaked on Nutrient agar (NA) and incubated at room temperature for 48h in inverted position.Visual characters of single colonies like colour, texture and mucoidal nature were recorded.

3.5.2.2 Morphological characterization

Gram's staining

A clean glass slide was taken and single drop of sterile water is poured on to it. A loopful of bacterial inoculum was taken (48h old culture) and a thin smear is prepared by thorough mixing. It then air dried and heat fixed followed by flooding with crystal violet (primary stain) for 30 sec. Then washed under tap water and again flooded with iodine solution for 1 min. followed by a wash with decolourizer (95 per cent alcohol). After this slide was taken washed under tap water and counterstained with safranin for 20 seconds. It was then washed again under running tap water and air dried. Then observed under microscope at 400X and 1000X magnification.

Scanning electron microscopy

Three dimensional imaging of bacterial isolate using Scanning electron microscopy (VEGA3 TESCAN) was conducted at Central Instrumentation Laboratory, College of Veterinary and Animal Sciences, Mannuthy, Thrissur.

Protocol

1. A loopful of 48h old culture (bacteria or yeast) were suspended in 1.5ml of phosphate buffer saline (PBS) of pH 7.0 in centrifuge tubes
2. The samples were centrifuged at 6000 rpm for 5 min.
3. Supernatant was discarded and 1.5ml of PBS was added followed by a centrifugation at 6000 rpm for 5 min.
4. Supernatant was discarded and 4µl of sample was smeared on each stub and sputter coated with gold particles at vaccum ($8x10^{-2}$mBar) and Nitrogen gas is used for creating vaccum to sputter coat the bacterial or yeast cells for 30 min.
5. This samples were taken after 30 min and kept inside scanning electron microscope for visualizing the cells.

3.5.3 Cultural and morphological characterization of antagonistic phylloshere yeast

Same as above (3.5.2).

3.6 MOLECULAR CHARACTERIZATION OF ANTAGONISTIC PHYLLOSPHERE MICROBES

3.6.1 Molecular characterization of antagonistic phyllosphere fungi

Same as 3.2.3

3.6.2 Molecular characterization of antagonistic phyllosphere bacteria

The promising phyllosphere isolates KPCSB1 and MUCSB1 were subjected to molecular characterization in order to identify them up to the species level. The aim of study was to confirm the results of cultural and morphological characters already done in (3.5.2). The bacterial isolates were characterized by 16S rDNA sequencing. This experiment was done at Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram. The bacteria were grown in nutrient agar slants were used for isolation and purification of genomic DNA from bacteria.

3.6.2.1 Genomic DNA Isolation from Bacteria

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel).

- A part of culture is taken in a microcentrifuge tube and 180 µl of T1 buffer and 25 µl of proteinase K was added and incubated at 56°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 minutes.
- 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 and 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

The quality of the DNA isolated was checked using agarose gel electrophoresis. 1µl of 6X gel-loading buffer (0.25 per cent bromophenol blue, 30 per cent sucrose in TE buffer pH-8.0) was added to 5µl of DNA. The samples were loaded to 0.8 per cent agarose gel prepared in 0.5X TBE (Tris-Borate-EDTA) buffer containing 0.5 µg/ml 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 and the image was captured under UV light using Gel documentation system (Bio-Rad).

The PCR amplification of 16S rRNA gene was performed in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems). Information on the primers used for the amplification reaction, composition and volume of PCR mix and amplification profile is given in Table 3.7 and 3.8 respectively.

Table 3.7 Components of PCR mix for amplification reaction

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

Table 3.8 Details of primers used for bacterial genomic DNA identification

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

3.6.3 PCR amplification profile:

95ºC-5.00 min

95ºC- 30 sec

60ºC- 40 sec }35 cycles

72ºC- 60 sec

72 ºC- 7.00 min

4ºC - ∞

3.6.2.3 *In silico* analysis of 16S- rRNA sequences

The sequence analysis was carried out using blastN search against NCBI nr database (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to obtain the best aligned sequences.

3.6.3 Molecular characterization of antagonistic phyllsophere yeast

Same as fungi (3.6.1)

3.7 INDUCTION OF SYSTEMIC RESISTANCE IN COWPEA BY ANTAGONISTIC PHYLLOSPHERE MICROORGANISMS

A pot culture experiment was conducted during September 2021 to October 2021 in the rain shelter of the Department of Plant Pathology, College of Agriculture, to study the systemic resistance induced by the promising five phyllosphere isolates against the anthracnose of cowpea (Plate 3.8).

The details of the experiment are as follows.

Design : CRD

Replication : 3

Number of seedlings in each replication : 2

Treatments : 7

Treatment details:

T_{1}- CKDSF1

T_{2} - KVKSF2

T_{3} - KPCSB1

T_{4} - MUCSB1

T_{5} - NKCSY1

T_{6} - Control

T_{7}- Absolute control

Here,

CKDSF1- Chirakkekode Stem Fungi 1

KVKSF2 – KVK Stem Fungi 2

KPCSB1 – Kallingalpadam Cowpea Stem Bacteria1

MUCSB1 – Mulleria Cowpea Stem Bacteria1

NKCSY1 – Natika Cowpea Stem Yeast 1

3.7.1 Inoculum preparation and challenge inoculation with the pathogen

Seven day old culture of pathogen was grown on neopeptone glucose agar in Petri plates and incubated for seven days. The mat of mycelium with spores was scrapped off from the plate and suspended in 100ml sterile water. Concentration of the inoculum was then adjusted to 10^6cfu/ml (Shiny *et al*., 2015). Cowpea seedlings were challenge inoculated by spraying in different treatments.

3.7.2 Estimation of defence related enzymes

The activity of defence related enzymes such as peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) were estimated on 0, 3, 5, 7 days after inoculation (DAI) by spectroscopy.

Leaf sample of 500mg from each treatment was weighed out and homogenized in 10mM sodium phosphate buffer using precooled pestle and mortar. The crude sap was centrifuged at 12000 rpm for 20 minutes at 4°C. The supernatant was collected in microcentrifuge tubes and stored at -20°C .This extract is used for the estimation of PO, PPO and PAL.

3.7.2.1Peroxidase (PO) activity

Crude extract (50ul) prepared from treated leaves (3.5.2) was added to one ml of 10mM sodium phosphate buffer (pH 6.0). It was mixed with pyrogallol (1ml) and finally, the substrate, H_2O_2 (one per cent) was added. Initial rate of increase absorbance was measured for 5 minutes at 1 minute interval at 436 nm.The peroxidase activity obtained was expressed as units of PO min^{-1} g^{-1} fresh tissue (Rathmell and Sequeria, 1974).

3.7.2.2 Polyphenol oxidase (PPO) activity

Crude extract (200ul) prepared from treated leaves (3.3.2) was added to 700µl of sodium phosphate buffer (pH 6.0) and it was mixed with 0.2M catechol (100µl). Initial rate of of increase absorbance was measured for 5 minutes at 420 nm. Polyphenol oxidase activity was expressed as units of PPO min^{-1} g^{-1} fresh tissue (Mayer and Harel, 1979).

Plate 3.8 Pot culture experiment to study the effect of phyllosphere antagonists on induced systemic resistance in cowpea

Plate 3.9 Preparation of culture filtrate for *in vitro* evaluation of secondary metabolites of phyllosphere antagonists against *Colletotrichum siamense*

Broth culture

Filtration

Centrifugation

Sterilization by Millipore filter

3.7.2.3 Phenylalanine ammonia lyase (PAL) activity

Crude extract (500ul) prepared from treated leaves (3.3.2) was added to500µl of 0.5M Tris HCL buffer (pH 8.5) and to this 0.15M L-Phenylalanine (500µl) was added and incubated at 37°C for 60 minutes. The reaction was stopped by adding 1M trichloro acetic acid (TCA)(500µl) and incubated at 40°C for 5 minutes and centrifuged to remove particles.The control tube contained L- Phenyl alanine added after TCA. The standard was prepared with different concentrations of transcinnamic acid and the absorbance was read at 270 nm in a UV visible spectrophotometer. Phenylalanine ammonia lyase activity was expressed as µmol of transcinnamic acid formed g^{-1} fresh tissue (Brueske, 1980).

3.8 EFFECT OF SECONDARY METABOLITES OF THE PHYLLOPSHERE MICROORGANISMS ON *Colletotrichum siamense*

The effect of secondary metabolites of the phyllopshere microorganisms on *Colletotrichum siamense* was evaluated under *in vitro* conditions.

3.8.1 Preparation of culture filtrate

The phyllosphere isolates were inoculated separately in 100ml of potato dextrose broth (PDB) (for fungus & yeast), nutrient broth (for bacteria) and incubated at 28±2°C for 21 days. The cell free culture filtrate of the isolates was prepared by removing the mycelium and other cells by filtering first through double layered filter paper (fungus) and centrifugation to remove pellets of bacterial and yeast cells (for bacteria and yeast) and then passing through bacterial proof filter of pore size 0.22 µm (Chandrakala *et al*., 2012) (Plate 3.9).

3.8.2 *In vitro* evaluation of secondary metabolites of phyllosphere microorganisms against *Colletotrichum siamense*

Mycelial disc of (8mm) pathogen was placed on 5 per cent culture filtrate amended PDA plate. The pathogen on PDA without the filtrate served as control. Observations on the growth of the pathogen were recorded.

3.9 *IN VITRO* EVALUATION OF ANATGONISTIC PHYLLSOPHERE MICROBES ON GROWTH PROMOTION IN COWPEA

An *in vitro* experiment was conducted to assess the effect of five selected phyllosphere antagonistic isolates (from experiment 3.3) on seed germination and seedling vigour.

The details of this experiment are as follows

Design : CRD

Number of treatments: 11

Number of replication : 3

Number of seeds per replication : 2

Cowpea variety used: Anashwara

Method of application: Seed treatment

The treatment details are given below.

Table 3.9 Treatments used for evaluation of seed vigour

Treatment	Isolate / Treatment
T_1	CKDSF1
T_2	KVKSF2
T_3	KPCSB1
T_4	MUCSB1
T_5	NKCSY1
T_6	*Trichoderma* sp. (@2% KAU)
T_7	*Pseudomonas fluorescens*(@ 2% KAU)
T_8	Hexaconazole @0.1%
T_9	Mancozeb @0.25%
T_{10}	Carbendazim + Mancozeb (@ 0.2%)
T_{11}	Control

3.9.1 Preparation of inoculum

Phyllosphere bacterial isolates were multiplied in nutrient broth. After 48h of incubation, the bacterial cells were taken and dispersed in sterile water so as to have 10^7cfu ml^{-1}. Fungal isolates the cultures were grown in PDB (Potato dextrose broth) for seven days and the spore suspension (10^7cfu $ml^{-1)}$ was made with sterile water.

3.9.2 Application of treatments and sowing

Cowpea seeds were treated by soaking them in different treatments for 30 min and the seeds treated with sterile water served as control. Treated seeds were placed on plain agar and kept under room temperature for germination. Observations were recorded.

3.10 *IN VIVO* EVALUATION OF ANATGONISTIC PHYLLSOPHERE MICROBES AGAINST ANTHRACNOSE DISEASE

Five selected antagonistic phyllosphere isolates including two fungi, two bacteria and one yeast were subjected to screening on *in vivo* conditions under rain shelter to understand their efficacy against the pathogen on cowpea plants (Plate 3.10).

3.10.1 Field experiment to evaluate of antagonistic phyllosphere microbes against *Colletotrichum siamense*

A field experiment was conducted to evaluate antagonistic phyllosphere isolates against the cowpea anthracnose disease in the rain shelter of Dept. of Plant Pathology, College of Agriculture, Vellanikkara, (Table 3.7).

The details of field experiment are,

Design : RBD

No. of treatments : 11

No. of replications : 3

Cowpea variety : Anashwara

Spacing : 60x45 cm

Plot size : 3m x 1m

3.10.2 Field preparation

Experimental plots were prepared by ploughing followed by levelling. Raised beds of 3m length, 1 m width and 25 cm height were taken after lime and FYM application. Bed to bed spacing was 40 cm.

3.10.3. Sowing

Inoculum for seed treatment was prepared and seeds were treated as explained in (3.4.1 & 2) used for sowing. Treatments were allocated randomly without repetition in same row. Treated seeds were sown according the layout design. Seeds treated with sterile water served as control. Seeds were sown at 60 x 45 cm spacing and 2 cm depth. All cultural operations except fungicidal application were carried out as per the Package of Practices Recommendations, Crops (KAU, 2016).

3.10.4 Treatment application

Plants were challenge inoculated with the pathogen at one month after planting (MAP). At the onset of the disease, treatment sprays were given three times at 15 days interval and observations (symptoms, per cent disease incidence, per cent disease severity, biometric characters and yield) were recorded.

3.10.5 Study on seed borne nature of the pathogen

Seeds from infected pods of plants in control were collected and placed on Petri dish containing PDA and incubated for 3 to 4 days at 28+/- 2^{o}C. The growth of pathogen was observed and characters were studied.

3.11 STATISTICAL ANALYSIS

Analysis of variance was performed on the data collected in various experiments using web agri-stat package (WASP 2.0). Transformations of the resultant data were done if required.

Plate 3.10 Views of field experiment

Results and Discussion

4. RESULTS AND DISCUSSION

The research on 'Phyllosphere microorganisms for the management of anthracnose disease of cowpea (*Vigna unguiculata* (L.) Walp.)' was carried out in the Department of Plant Pathology, College of Agriculture, Vellanikkara, during the period 2019-2021. The results of experiments conducted as part of the research are presented here and discussed in the light of available literature.

4.1 COLLECTION, ISOLATION AND IDENTIFICATION OF THE PATHOGEN ASSOCIATED WITH COWPEA ANTHRACNOSE DISEASE

Anthracnose of cowpea, popularly known in Malayalam as "karimban kedu or karivally" is one of the most important diseases affecting yield of the crop in Kerala. The causal organism has been reported as *C. lindemuthianum* (Kumar, 1999). However, there are recent reports suggesting very high variability in the population of the fungus. As part of the present investigation, purposive sampling surveys were conducted during the period from February 2020 to September 2021 in cowpea growing areas of 10 locations of Thrissur, two locations of Kasaragod and one location each from Palakkad, Malappuram and Kozhikode (Plate 4.1). Diseased and healthy samples of cowpea plants were collected from each location. The symptoms varied among locations and cultivars of the crop (Plate 4.2). The disease severity varied from 36 to 86 per cent. The highest per cent disease incidence was observed at cowpea field in Vidya Nagar (High Tech Research and Training Institute) and Bosco Nagar near Vellanikkara (100 per cent) (Table 4.1).

Symptoms of cowpea anthracnose observed during survey include, pinkish brown spindle-shaped sunken lesions on main stems and vines lead to girdled appearance, dark necrotic spots on stems, brown spots followed by shot-hole appearance on leaves and water-soaked lesions on collar regions. In some areas, plants with collar infection caused yellowing and withering of the vines, which subsequently lead to secondary infections. Hence, in anthracnose-affected cowpea fields, mixed infections with other diseases were also observed. Surveys in the districts of Palakkad and Thrissur yielded two types of leaf spots. Different types of circular to elongated sunken lesions on stems and vines were observed in the remaining samples (Plate 4.2).

Table 4.1 Per cent disease incidence and severity of anthracnose of cowpea at different locations

Sl no.	Location	Disease symptoms observed	PDI %	PDS %
1.	Nemmara (Palakkad)	Brown spot (4 mm size) on leaf	75	65
2.	Natika (Thrissur)	Eye shaped sunken lesions on stem	86	78
3.	Thalakulathur (Kozhikode)	Black round to oval spots on stem	20	50
4.	Kallingalpadam (Thrissur)	Eye shaped brown sunken lesions on stem	95	48
5.	Madannur (Thrissur)	Dark brown spots on stem	70	52
6.	Pandallur (Thrissur)	Spindle shaped sunken lesions on stem	98	95
7.	Dept. of Vegetable science, COA, Vellanikkara (Thrissur) Plot 1	Water soaked lesions on collar region and yellowing of plants	86	46
8.	" " Plot 2	Dark brown sunken lesions on collar region,stem and petioles	72	55
9.	Mulleria (Kasaragod)	Black circular sunken spots on main stem and branches	35	43
10.	High tech research and training institute, Vidyanagar, (Thrissur)	Brown to pinkish spindle shaped, elongated sunken lesions on vines and leaves	100	85
11.	Ollukkara(Thrissur)	Brown and black oval sunken spots on stems	90	78
12.	Bosco Nagar (Vellanikkara – Tens) (Thrissur)	Dark brown elongated sunken lesions on stems, vines, petioles, leaf veins and pods	100	86
13.	Ariyallur (Malappuram)	Dark circular necrotic spots on main stem and branches of the vine	65	36
14.	Mannuthy (Thrissur)	Brown to pinkish eye shaped lesions and shot-hole symptoms on leaves	70	65
15.	Ukkinadka (Kasaragod)	Dark brown eye shaped sunken lesions on stems	40	23

PDI – Per cent disease incidence, PDS- Per cent disease severity

Plate 4.1 Views of locations surveyed

Madakkathara

Mannuthy

Vidyanagar

Ukkinadka

Natika

Panthallur

Bosconagar

Kallingalpadam

Vellanikkara

Plate 4.2 Cowpea anthracnose symptoms observed during survey

1) **<u>Dark brown elongated sunken lesions on stems, petiole and leaf veins</u>**

Vidyanagar

Bosconagar

Ollukkara

Natika

Panthallur

Vellanikkara (plot 1)

Vellanikkara (plot 2)

Madannur

2) Dark oval lesions on vines

Thalakkulathur

Badiadka

Ariyallur

Ukkinadka

3) Leaf spots

Mannuthy

Nenmara

It was found that, eventhoughthe disease spread is favoured by high atmospheric humidity and low temperature (Singh *et al*., 1997; Kumar, 1999), the disease incidence is seen throughout the year in both pole and bushy types. Later in the study, involvement of different fungi and different strains of the pathogen was observed. Hence, this might have attributed to the variation in season of occurrence.

4.1.2 Isolation of the pathogen

The pathogen causing anthracnose disease in cowpea was isolated from naturally infected cowpea plants.The fungi associated with the symptoms were isolated and brought into pure culture following standard procedures. A total of 21 fungal isolates were obtained from 15 different locations of survey. Pathogenicity of the organism was proved by inoculation on healthy cowpea leaves and stems. Isolates which did not produce typical symptoms on artificial inoculation were discarded. This includes *Fusarium* sp., *Curvularia* sp., *Corynespora* sp., *Diplodia* sp. *etc.* Out of the 21 isolates collected, only six could produce the symptoms on artificial inoculation. These were re- isolated and the cultural and morphological characters found to be same as that of the original one, hence confirming the pathogenicity. These fungi were given codes VHT, BNT, OLR, UDK, MLA and ALR (Table 4.1) (Plate 4.3).

Plants with severe infection yielded different types of organisms upon isolation. Symptoms on natural as well as artificial inoculation varied among different isolates, suggesting involvement of various fungi and existence of variability within the population of the pathogen. The other fifteen isolates could not proved pathogenicity, this may due to association of these isolates as secondary intruders or saprophytes so they could not produced symptoms on artificial inoculation.

Table 4.2 Symptoms produced by different isolates on artificial inoculation

Sl No	Location	Designated code	Symptoms produced due to artificial inoculation	Time taken for infection (days)
1.	Vidya Nagar (Thrissur)	VHT	Darkening on stem, petiole with yellow hallow	3
2.	Ollukkara (Thrissur)	OLR	Dark necrotic spindle shaped spots on leaf veins, stem with yellow hallow	3
3.	Bosco Nagar (Thrissur)	BNT	Brown discolouration on stem and leaf veins	3
4.	Ariyallur (Malappuram)	ALR	Brown elongated lesion on leaf petiole	4
5.	Mulleria (Kasaragod)	MLA	Yellowing at the point of inoculation, shot-hole symptom	4
6.	Ukkinadka (Kasaragod)	UDK	Dark discolouration and yellow hallow on leaf veins	3

4.2 CHARACTERIZATION OF PATHOGENS ASSOCIATED WITH ANTHRACNOSE OF COWPEA

The isolates VHT, BNT, OLR, UDK, MLA and ALR, which are proved to be pathogenic on cowpea producing anthracnose symptoms were purified and characterized. The cultural and morphological characters of pathogens are given in Table 4.3 and 4.4.

4.2.1. Cultural and morphological characterization of VHT

Greyish white fluffy aerial mycelia with olivaceous green tinge developed from the infected tissue bits, later produced off white to greyish cottony mycelial growth and also observed off white to brown pigmentation on the reverse side of the Petri dish. Furthermore, the formation of orange coloured conidial masses was detected. The growth rate was 0.9 cm per day and taken 7 to 8 days for full growth (9cm) in PDA plate. Under microscope, the fungal tissue was observed to study the morphological characters. Production of fruiting bodies (acervuli with setae ranging from 71.9–81.9 μm length) and

Plate 4.3 Symptoms produced by different pathogens on artificial inoculation

Vidya nagar (VHT)

Bosconagar (BNT)

Ollukkara (OLR)

Ukkinadka (UDK)

Ariyallur (ALR)

Badiadka (BDK)

bullet or cylindrical shaped conidia (6.2-7.4 x 2.4- 3.1 µm size) having oil globules were observed under microscope (1000X) (Plate 4.4).

4.2.1.2 Cultural and morphological characterization of BNT

Growth of greyish aerial mycelium radiating from infected tissue bits was observed on PDA. Later, white to greyish white fluffy mycelial growth and dark greyish to black radial lines on the reverse side of the Petri dish was seen. Similar to VHT, it also produced pale yellow coloured conidial masses upon maturation. The growth rate was 1.05 cm per day and took 6 to 7 days for full growth (9cm). Acervuli with setae, ranging from 41.5–53.5 µm in length and bullet or cylindrical shaped conidia (4.8- 7.1 µm x 1.8- 2.8 µm size) having oil globules were observed under microscope (1000X) (Plate 4.5).

4.2.1.3 Cultural and morphological characterization of OLR

The pathogen produced off-white mycelium and dark grayish to black pigmentation on the reverse side of the Petri dish. The growth rate was 1.08 cm per day and it took 6 to 7 days for full growth (9 cm) in the PDA plate. Upon maturation, production of pale yellow conidial masses are noticed. Acervuli with setae ranging from 55.3–61.8 µm in length, bullet shaped conidia (7.9- 8.7 x 2.1- 3.2 µm size) having oil globules were produced (Plate 4.6).

4.2.1.4 Cultural and morphological characterization of UDK

The pathogen UDK produced grey mycelium and greyish to dark radial lines on reverse side of the Petri dish. The growth rate was 1.1 cm per day and it took 6 to 7 days for full growth (9cm) in Petri dish. Acervuli with setae ranging from 65.5 – 74.9 µm length and bullet shaped conidia (7.7- 8.3 x 2.0- 3.1µm size) having oil globules were produced (Plate 4.10) and it took 6 to 7 days for full growth (9 cm) in Petri dish (Plate 4.7).

4.2.1.5 Cultural and morphological characterization of MLA

The pathogen MLA produced olive green to greyish mycelial growth in the early stages and metallic black hard pustules remain, once mycelia has faded. Under microscopic examination the hard bodies are confirmed as ‘pycnidia’ of the pathogen which is olive

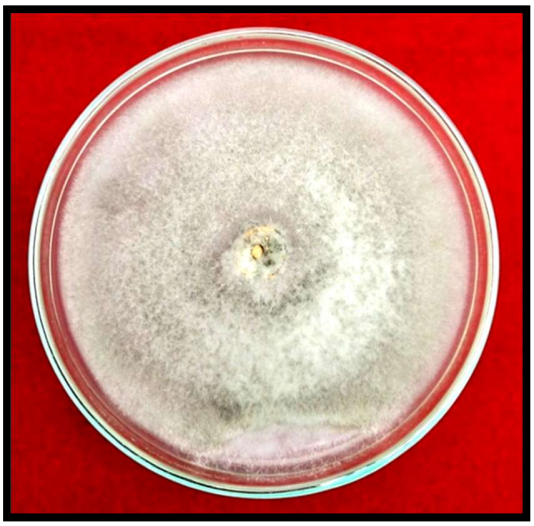
green to dark brown with appressed texture having small, predominantly globose pycnidial bodies (Chobe *et al*., 2020) and spores are small and bullet shaped (4.8 – 5.3 x 1.8- 2.2µm size).The growth rate was 1.1cm per day and it took 5 to 6 days for full growth (9 cm) in Petri dish (Plate 4.8).

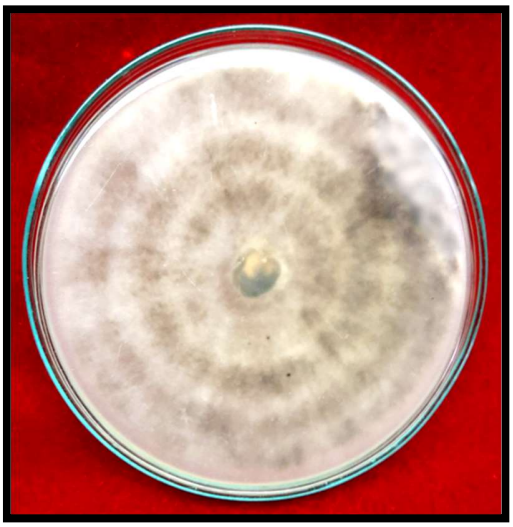
4.2.1.6 Cultural and morphological characterization of ALR

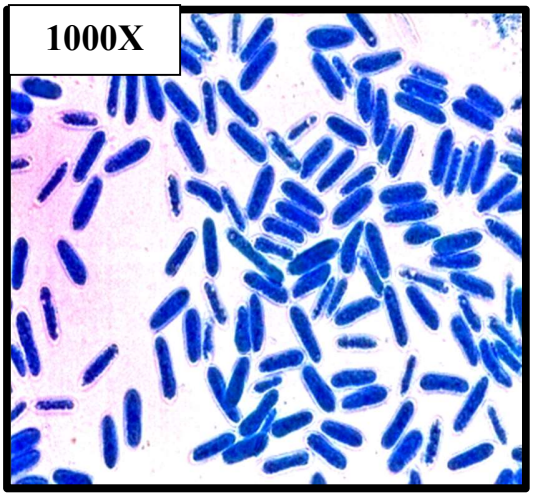
The pathogen ALR was differed from all other isolates. It produced dark greyish flattened mycelium later turn dark black colour. On reverse side of Petri dish black colour pigmentation was seen. Conidia formed on the conidiophores, they are cylindrical straight to slightly curve with the second cell larger than other cells and both ends of the cells are sub hyaline and other cells are brown coloured (5.13–10.41µm X 3.25– 4.53 µm). It took 3 to 4 days for full growth (9 cm) in Petri dish and growth rate was 1.28cm per day (Plate 4.9).

The cultural and morphological characters of the isolates VHT, BNT, OLR, UDK are found to be more or less similar. Generally, they produced greyish mycelium which later produced pale yellow to orange conidial masses. They produced cylindrical or bullet shaped hyaline conidia and acervuli. Hence these four isolates belong to genus *Colletotrichum* (Jha *et al*., 2012). Based on the morphological features, the isolates VHT, BNT and OLR were tentatively identifed as species belong in *C. gloeosporioides* species complex (Weir *et al*., 2012). The pathogen MLA produced tiny, numerous bullet-shaped conidia, it was mistaken for the fungus "*Colletotrichum*". But instead of acervuli, it produced pycnidia, indicating that it do not belong to the genus *"Colletotrichum."* It could be a member of the genus*"Phoma"*.The cultural and morphological characters exhibited by the isolate MLA was similar to *Phoma* sp., as it produced brown tinged olivaceous green mycelium and tiny bullet or oval shaped spores and pycnidia (Chobe *et al*., 2020).The isolate ALR showed the characters of *Curvularia* sp. It produced dark greyish flattened mycelium and cylindrical straight to slightly curved (second cell) conidia on conidiophoresand the growth rate of other pathogens such as VHT, BNT, OLR, UDK and MLA are more or less similar (0.9- 1.1cm per day) but, the pathogen ALR was faster in growth under room temperature (28+/-2ºC)(Huang *et al*., 2005).

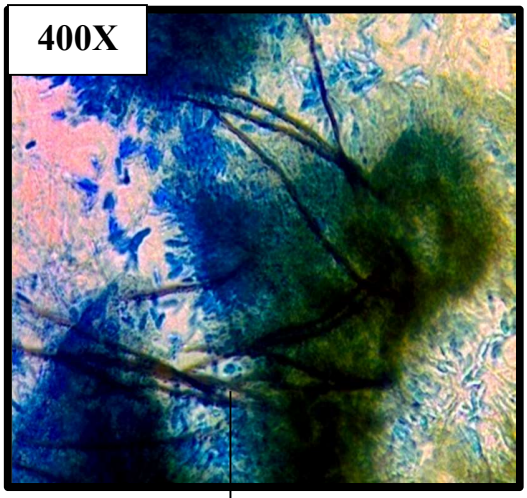
Plate 4.4 Culture of VHT with conidia





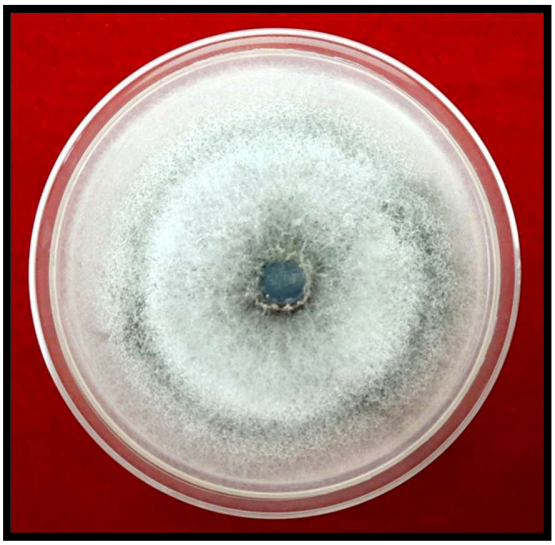


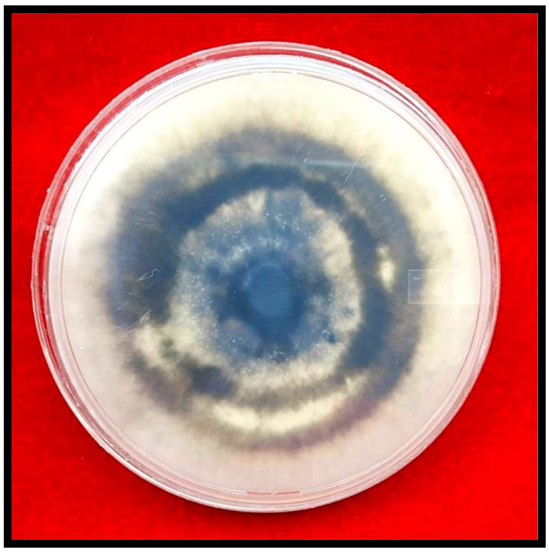
Conidia (6.2- 7.4 x 2.4-3.1µm)

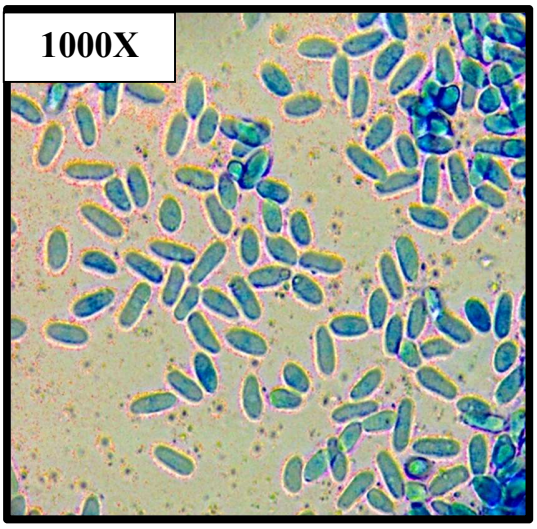


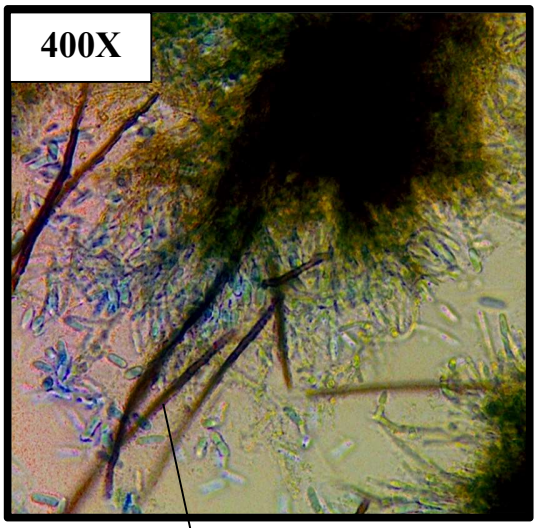
Acervuli with setae

Plate 4.5 Culture of BNT with conidia





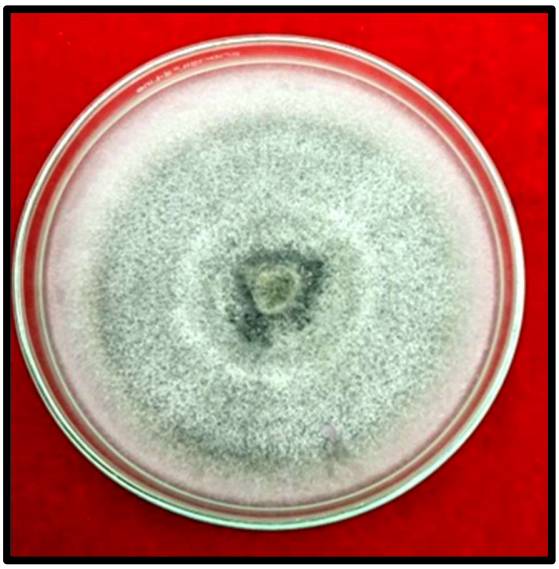


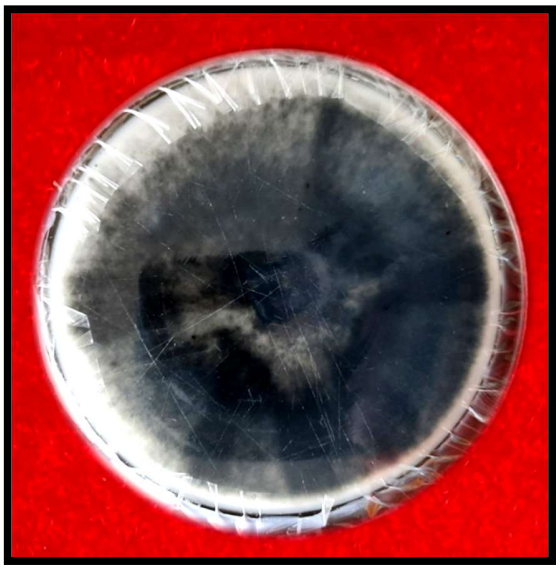


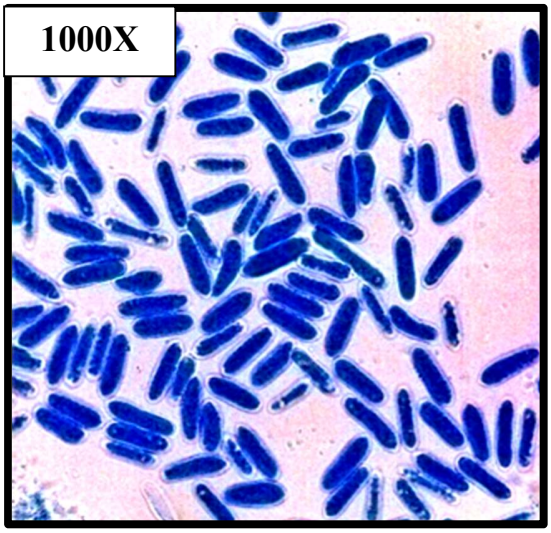
Conidia (4.8- 7.1 x 1.8- 2.8 µm)

Acervuli and setae

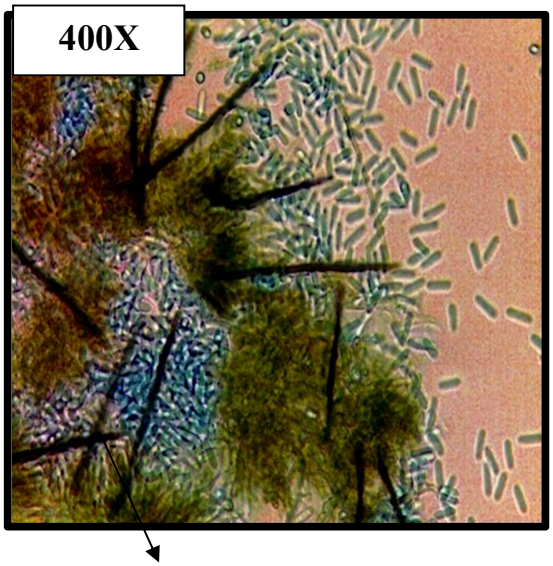
Plate 4.6 Culture of OLR with conidia





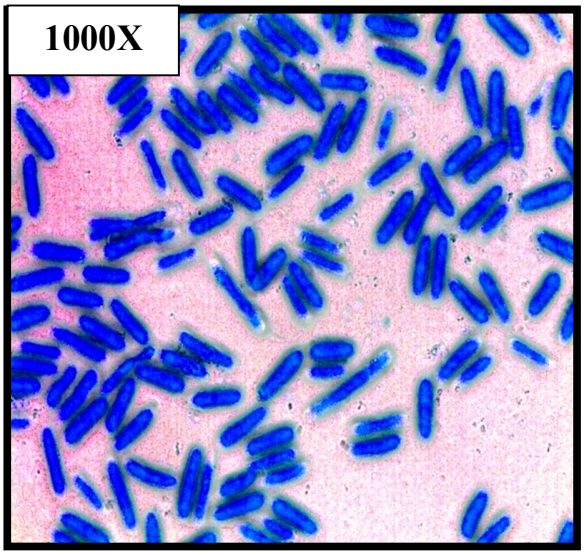


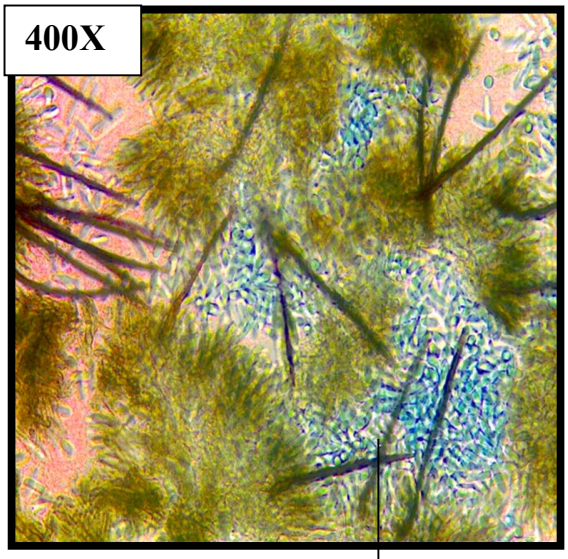
Conidia (7.9-8.7 x 2.1- 3.2 µm)



Acervuli with setae

Plate 4.7 Culture of UDK with conidia

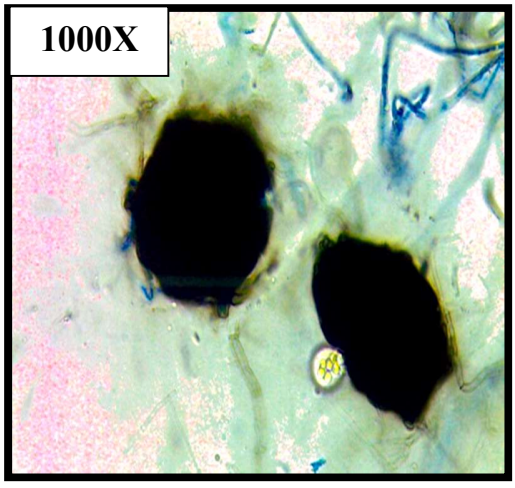




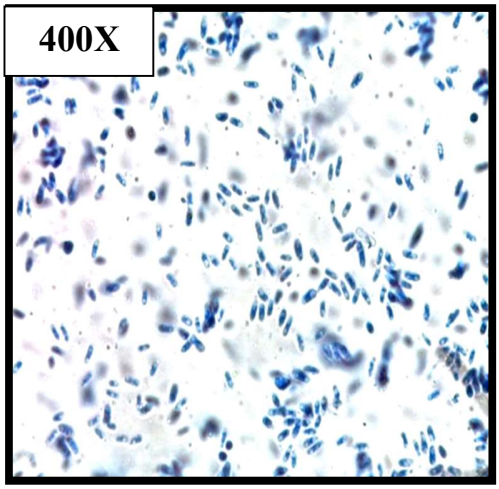
Conidia (7.7- 8.3 x 2.0- 3.1µm)

Acervuli with setae

Plate 4.8 Culture of MLA with conidia

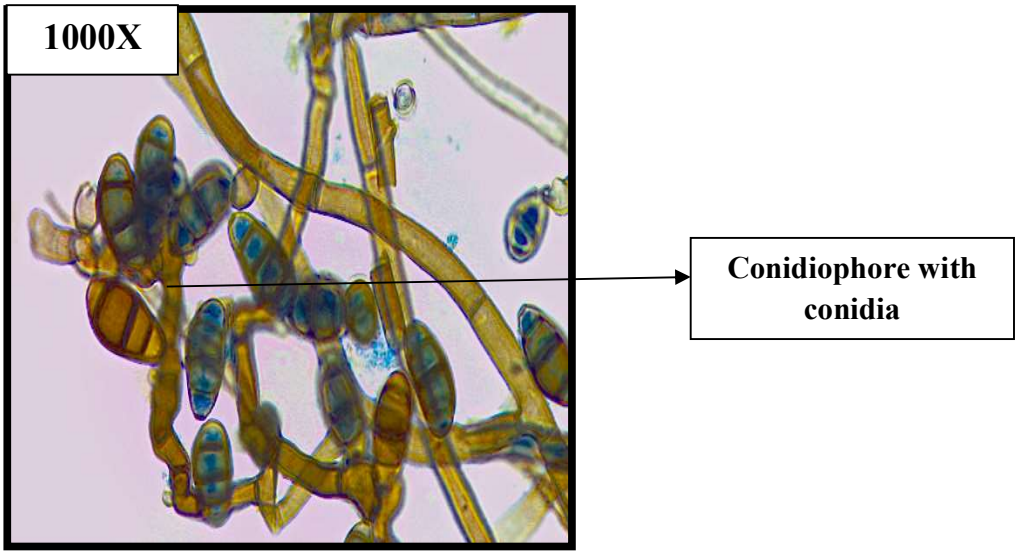


Conidia (4.8-5.3 x1.8- 2.2 µm)



Pycnidium

Plate 4.9 Culture of ALR with conidia



Conidia (5.13–10.41x3.25– 4.53µm)

Table 4.3 Morphological characterization of pathogens

Isolate	Morphological characters			
	Hyphae	**Shape and colour of conidia**	**Conidiophore features**	**Conidial dimensions**
VHT	Hyaline, septate, branched	Cylindrical, hyaline	Simple, short, hyaline, cylindrical	6.2-7.4 x 2.4- 3.1 µm
BNT	Hyaline, septate, branched	Cylindrical, hyaline	Simple, short, hyaline, cylindrical	4.8- 7.1 x 1.8- 2.8 µm
OLR	Hyaline, septate, branched	Cylindrical, hyaline	Simple, short, hyaline, cylindrical	7.9- 8.7 x 2.1- 3.2 µm
UDK	Hyaline, septate, branched	Cylindrical, hyaline	Simple, short, hyaline, cylindrical	7.7- 8.3 x 2.0- 3.1µm
MLA	Hyaline to brown, septate, branched	Oval to bullet shaped Hyaline	Phialidic conidiogenous cell, with short neck, hyaline	4.8 – 5.3 x 1.8- 2.2 µm
ALR	Brown, septate, branched	Cylindrical straight to slightly curve, second cell larger than other cells and both ends of the cells are sub hyaline and others are brown	Septate, erect / cuved, light brown, geniculate close to apex	5.13–10.41x 3.25– 4.53µm

Table 4.4 Growth rate of pathogens on PDA

Sl no.	Pathogens	Mean colony diameter (cm) Days after incubation									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**No. of days taken for full growth**	**Growth rate**
1	VHT	1.2	2.1	3.6	4.3	5.4	6.5	7.8	9.0	8	0.9
2	BNT	1.6	2.9	4.1	5.7	6.3	7.4	9	-	7	1.05
3	OLR	1.4	2.6	3.3	4.6	5.8	6.8	7.9	9.0	8	1.08
4	UDK	1.2	2.0	3.5	4.7	6.6	7.9	9.0	-	7	1.1
5	MLA	1.1	2.6	3.8	5.0	6.2	7.8	9.0	-	7	1.1
6	ALR	1.6	3.5	6.8	8.5	9.0	-	-	-	5	1.28

This study revealed that, various species under the genus *Colletotrichum* are responsible for the disease and they are similar in cultural and morphological characteristics. However, they showed slight variation with regard to the size of conidia and length of setae. In the *Colletotrichum* patho- system, it is known that different species could cause anthracnose on the same host (Enyiukwu *et al*., 2014). As there is variability in the morphological characters of *Colletotrichum* sp. observed during the present study, it may be assumed that there are different species of the fungus causing anthracnose of cowpea. Another reason may be that the fungus is continuously evolving. It is also found that, *Colletotrichum* is not the only fungus infecting cowpea and producing typical anthracnose symptoms, in addition to *Colletotrichum*, *Phoma* sp., *Curvularia* sp. also produce anthracnose symptoms on cowpea. This is the first report of these two pathogens involved in anthracnose of cowpea.

4.2.2 MOLECULAR CHARACTERIZATION OF PATHOGEN

4.2.2.1 Molecular characterization of VHT (*Colletotrichum siamense*)

The BLASTn analysis of ITS sequences of the pathogen isolate VHT in NCBI nr database showed 99.53 per cent identity with *Colletotrichum siamense* isolate ALSKN-CG5 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (MT450691.1) with 99 per cent query cover. Along with this the sequence showed 99.53 per cent similarity with the accessions of the same pathogen (MN595959.1, KX227593.1, LC580220.1).

4.2.2.2 Molecular characterization of BNT (*Colletotrichum* sp.)

The BLASTn analysis of ITS sequences of the pathogen isolate BNT in NCBI nr database showed 99.77 per cent identity with *Colletotrichum* sp. AR3750 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (MT286216.1) with 100 per cent query cover.

4.2.2.3Molecular characterization of OLR (*Colletotrichum* sp.)

The BLASTn analysis of ITS sequences of the pathogen isolate OLR in NCBI nr database showed 99.69 per cent identity with *Colletotrichum* sp. AR3750 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (MT286216.1) with 100 per cent query cover.

4.2.2.4 Molecular characterization of UDK (*Colletotrichum* sp.)

The BLASTn analysis of ITS sequences of the pathogen isolate UDK in NCBI nr database showed 98.96 per cent identity with *Colletotrichum plurivorum* small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal

RNA gene, partial sequence (MT351124.1) with 79 per cent query cover. Since the query coverage is very less species level cannot be confirmed.

4.2.2.5 Molecular characterization of MLA (*Ectophoma multirostrata*)

The BLASTn analysis of ITS sequences of the pathogen isolate BDK in NCBI nr database showed 99.20 per cent identity with *Ectophoma multirostrata*, CP PM01small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (MG897497.1) with 100 per cent query cover.

4.2.3.6 Molecular characterization of ALR (*Curvularia verruculosa*)

The BLASTn analysis of ITS sequences of the pathogen isolate ALR in NCBI nr database showed 99.62 per cent identity with *Curvularia verruculosa* strain JAD2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (MK120494.1) with 100 per cent query cover.

Table 4.5 *In silico* analysis of ITS sequences of pathogens

Isolate	Description	Max score (%)	e – value	Query coverage (%)	Identity percentage
VHT	*Colletotrichum siamense*	778	0.0	99	99.53
BNT	*Colletotrichum* sp. AR3750	2385	0.0	100	99.69
OLR	*Colletotrichum* sp. AR3750	2399	0.0	100	99.77
UDK	*Colletotrichum plurivorum*	1880	0.0	79	98.96
MLA	*Ectophoma multirostrata*	905	0.0	100	99.20
ALR	*Curvularia verruculosa*	1424	0.0	100	99.62

4.2.4 Phylogenetic analysis

The evolutionary relationship between *Colletotrichum siamense* (VHT) and other top hits obtained from BLASTn analysis in NCBI nr database revealed that the pathogen associated with cowpea anthracnose symptom VHT was more related to the accession of *C. siamense* ALSKN CG5 as well as *C. gloeosporiodes* isolate MR2 (MN595959.1)and distantly related to *C. plurivorum*. The evolutionary relationship between *Colletotrichum* sp. AR3750(BNT) and other top hits obtained from BLASTn analysis in NCBI nr database revealed that the pathogen associated with cowpea anthracnose symptom BNT was more related to the accession of *C. siamense* CPV3.6 (LC585220.1)and distantly related to *Colletotrichum* sp. AR3750. The evolutionary relationship between *Colletotrichum* sp. AR3750(OLR) and other top hits obtained from BLASTn analysis in NCBI nr database revealed that the pathogen associated with cowpea anthracnose symptom OLR was more related to the accession of *C. siamense* CPV3.6 (LC585220.1)and distantly related to *Colletotrichum* sp. AR3750. The evolutionary relationship between *C. plurivorum*(UDK) and other top hits obtained from BLASTn analysis in NCBI nr database revealed that the pathogen associated with cowpea anthracnose symptom UDK was more related to the accession of *C. plurivorum* HJ13 (MH318546.1)and distantly related to *C.plurivorum* QC15. The evolutionary relationship between *Ectophoma multirostrata*, CP PM01, (BDK) and other top hits obtained from BLASTn analysis in NCBI nr database revealed that the pathogen associated with cowpea anthracnose symptom BDK was more related to the accession of *C. plurivorum* HJ1, *Ectophoma multirostrata*, CP PM013 (MG897497.1)and distantly related to *Ectophoma multirostrata* Ach R3. The evolutionary relationship between *Curvularia verruculosa* strain JAD2, (ALR) and other top hits obtained from BLASTn analysis in NCBI nr database revealed that the pathogen associated with cowpea anthracnose symptom ALR was more related to the accession of *C. lunata* ERR 14-6 (MH443366.1)and distantly related to *C. verruculosa* PD30.

Among the six fungal isolates collected during sampling survey, four isolates (VHT, BNT, OLR, UDK) were confirmed as under the genus, *Colletotrichum*. Mega X software was used to analyse the evolutionary relationship of different *Colletotrichum* isolates obtained during the survey with other species of the same pathogen obtained

during BLASTn analysis in NCBI nr database. The evolutionary relationship of VHT, BNT, OLR and UDK with four accessions each of *Colletotrichum gloeosporioides*, *C. siamense*, *C. plurivorum* and *C. cliviicola* was analysed by constructing a neighbor-joining tree using Mega X. The phylogenetic tree revealed that all the accessions were related to each other and the species diversification study based on ITS sequencing alone was not possible as the different species of *Colletotrichum* were clustering together (Fig 4.1). Based on the analysis, it was confirmed that the three isolates VHT, BNT and OLR belonged to *C. gloeosporioides* species complex and UDK belongs to *Colletotrichum orchidearum* species complex.

In the present study, variability among pathogens causing anthracnose in cowpea was observed. The BLASTn analysis of ITS sequences of the pathogen isolates in NCBI website yielded various results. The isolate VHT identified as *Colletotrichum siamense* ALSKN CG5 with 99.53 per cent identity and 99 per cent query cover. Phylogenetic analysis of isolate VHT showed 100 per cent similar to both *C. siamense* ALSKN CG5 and *C. gloesporiodes* MR2. There is a report on infection of *C. gloeosporiodes* on cowpea (Barreto *et al*., 2007). Molecular characterization of the isolate VHT showed that it is more resembled to '*siamense*' species and it belongs to *C. gloeosporioides* species complex (Weir *et al.,* 2012). The isolate BNT identified as *Colletotrichum* sp. AR3750 with 99.69 per cent identity and 100 per cent query cover and OLR also identified as *Colletotrichum* sp. AR3750 with 99.77 per cent identity and 100 per cent query cover. *Colletotrichum* sp. AR3750 is classified under unclassified *Colletotrichum* group (Schoch *et al*., 2020). But the evolutionary relation of these isolates by phylogenetic analysis showed both BNT and OLR are more related to *C. siamense* CPV3.6. Hence, it is confirmed that the isolates VHT, BNT and OLR are different strains of *C. siamense* comes under *C. gloeosporioides* species complex and have more or less similar characters with the common ancestor(Weir *et al*., 2012). Till now, to our knowledge *C. lindemuthianum* is the common causal agent for cowpea anthracnose disease (Allen, 1983; Kumar, 1999; Rio *et al*., 2002; Pradhan *et al*., 2018; Satpathy and Beura, 2021) also some other species of *Colletotrichum* were also reported in the worldsuch as *C. gloeosporioides* f.sp. *aeschynomene* (Singh *et al*., 1997),*C. destructivum* (Adegbite and Amusa, 2008; Enyiukwu *et al*., 2014), *Colletotrichum fructicola*(Atghia *et al*., 2015), *C. dematium* (Emechebe and Florini, 1997; Enyiukwu,

Fig 4.1 Neighbor-joining phylogenetic tree constructed in Mega X showing relationship between various species of the genus *Colletotrichum*

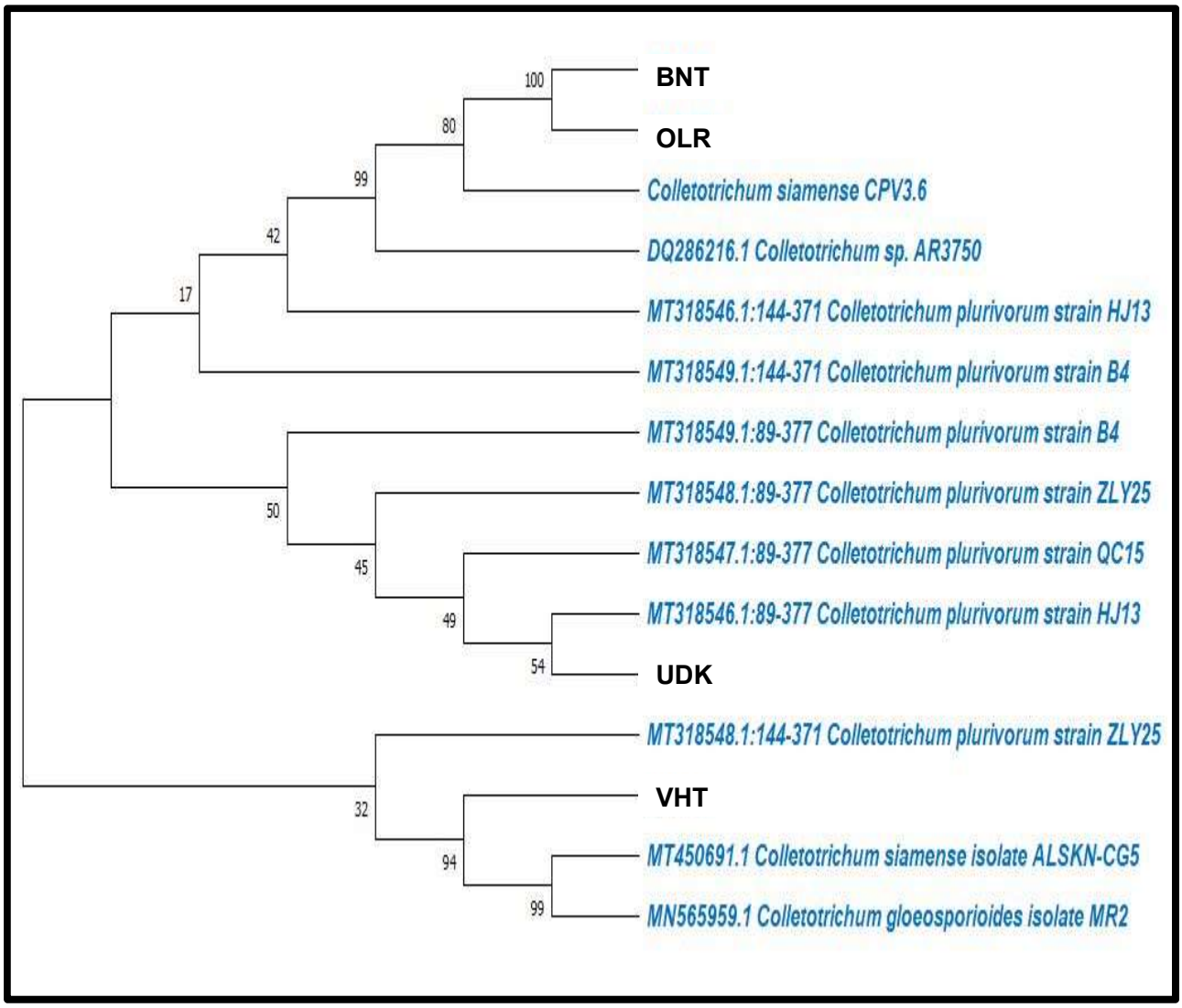


Fig 4.2 Phylogenetic tree of *Phoma* sp. by bootstrap method

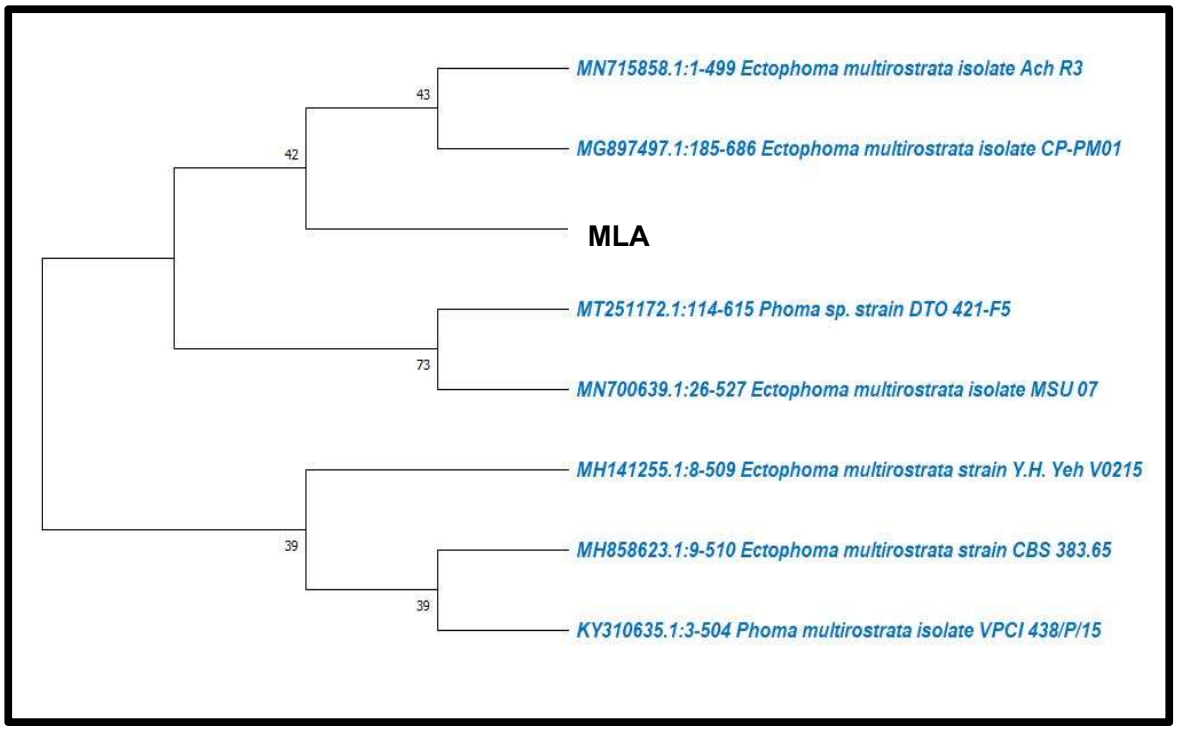
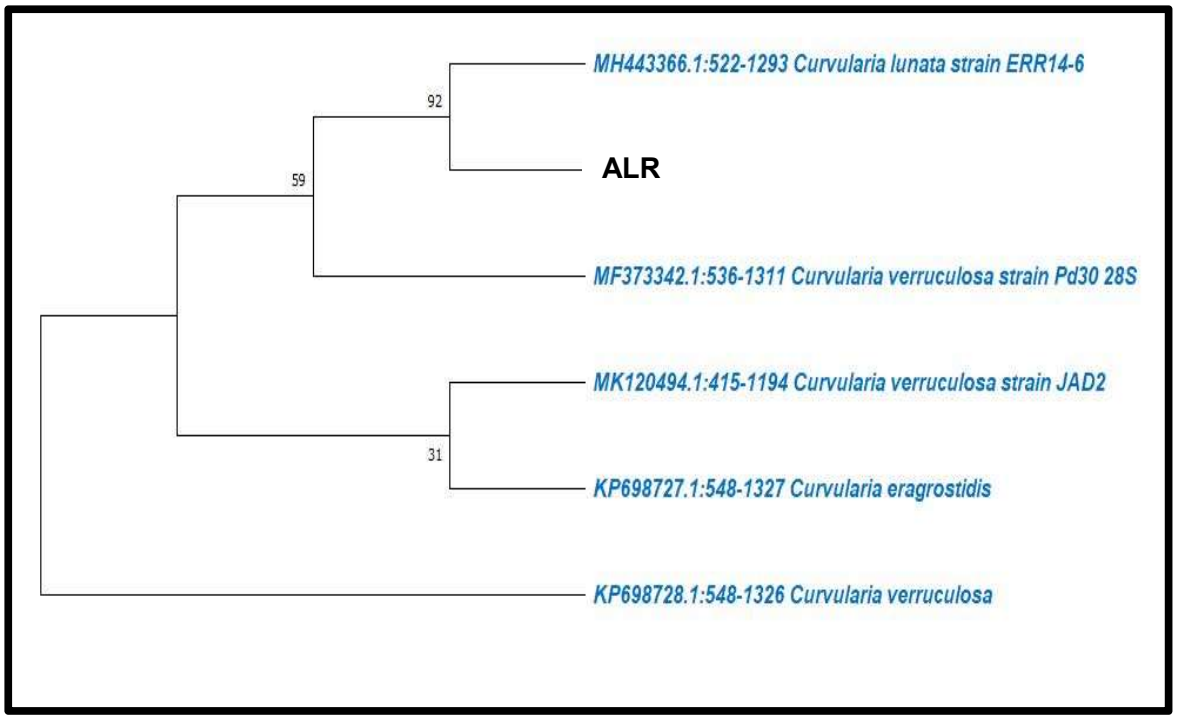


Fig 4.3 Phylogenetic tree of *Curvularia* sp. by bootstrap method



2017),*C. gloeosporioides, C. fragerie, C. dermatium* and *C. destructivum* (Enyiukwu, 2017).*C. gloeosporioides, C. fragerie* (Enyiukwu, 2017). In cowpea, anthracnose disease caused by *C. siamense* has been the first report and it is contradictory to many previous reports worldwide. There are other reports on *C. siamense* causing anthracnose in various crops such as citrus (Abhirami *et al*., 2019), onion (Herath *et al*., 2021). The isolate UDK showed similarity with *Colletotrichum cliviicola* with 98.96 per cent identity and 79 per cent query cover. Evolutionary relation of this isolate by phylogenetic analysis showed that is more related *C. plurivorum* isolate HJ13. Since, the query coverage is less than 90 per cent, confirmation is not feasible. Recently, C. *plurivorum* reported to cause soybean anthracnose (Barbieri *et al*., 2017) and the species belong to to the *C. orchidearum* species complex (Damm *et al*., 2019).Hence, from this study it is revealed that new species of the pathogen *viz.C. siamense* also responsible to cause anthracnose in cowpea.

During isolation of pathogens various fungal isolates were obtained which belong to the genus, *Fusarium, Curvularia, Phoma, Diploidea, Alternaria, Corynespora* etc. and during pathogenicity test *Phoma* (MLA) and *Curvularia* (ALR) established pathogenicity and others were discarded. The isolate MLA was identified as *Ectophoma multirostrata* isolate CP.PM01 from the molecular characterization and evolutionary relationship with *Ectophoma multirostrata* isolate CP.PM01 was also showed in phylogenetic analysis in Mega X software (Fig. 4.2).There is no evidence for the report of the pathogen *E. multirostrata* causing anthracnose in cowpea. Research evidence shows that it is also a first report of *E. multirostrata* to cause dry root rot in chickpea (Chobe *et al*., 2020).

The isolate ALR identified as *C. verruculosa* and evolutionary relationship with *Curvularia lunata* strain ERR 14-6 was showed in phylogenetic analysis in Mega X software (Fig. 4.3). *C. lunata* is a major leaf spot pathogen. There are many reports of cowpea leaf spot caused by *C. lunata* (Upadhyaya, 1980; Liu *et al*., 2010; Ekhuemelo *et al*., 2019). Very limited number of reports on stem infection of this pathogen. There is a report of stem blight disease caused by *C. lunata* (Msikita *et al*., 2007). Hence, it was a breakthrough in this research that, pathogens apart from *Colletotrichum* are causing anthracnose symptom in cowpea. These variability among the pathogens may be due to introduction of new species or migration, climate change and after effects of two floods in

Kerala and other factors (Chakraborty, 2013; Kumar and Verma, 2019; Thomas and Dinesh, 2020).

4.3 ISOLATION AND ENUMERATION OF PHYLLOSPHERE MICROFLORA ON COWPEA

Healthy plant parts of cowpea such as leaves, stems, pods, flowers collected during the survey were used for enumeration and isolation of phyllosphere microflora. The total phyllosphere microflora *viz.* fungi, bacteria, yeasts, fluorescent pseudomonads and actinomycetes were quantitatively estimated by serial dilution plating of plant washings. Actinomycetes could not be isolated from any of the samples collected. The population of phyllosphere microflora including fungi, bacteria, fluorescent pseudomonads, yeasts is given in the tables 4.6, 4.7, 4.8, 4.9 respectively. Population of microbes was estimated by counting the number of colonies of microbes with respect to area of the sample used for serial dilution and dilution factor.

4.3.1 Population phyllosphere fungi on cowpea

It was found that, the mean population of phyllosphere fungi is more in cowpea flowers (10.0 x 10^2cfu cm^{-2} area), followed by pods (7.11 x 10^2cfu cm^{-2} area), stems (6.44 x 10^2cfu cm^{-2} area) and leaves (3.37 x 10^2cfu cm^{-2} area) (Fig 4.4). Among fifteen locations surveyed, the highest population phyllosphere fungi obtained from samples, Chirakkekode 2 (66.1x 10^2cfu cm^{-2} pod area), followed by Natika (65.5 x 10^2 cfu cm^{-2} flower area), Natika (22.02 x 10^2cfu cm^{-2} stem area) and it was found that phyllosphere fungi ranks second highest among the phyllosphere microflora.

4.3.2 Population of phyllosphere bacterium on cowpea

It was found that, the mean population of phyllosphere bacteria is more in cowpea flowers (42.94 x 10^6cfu cm^{-2} area), followed by stems (23.42 x 10^6cfu cm^{-2} area), pods (5.37 x 10^6cfu cm^{-2} area) and leaves (3.04 x 10^6cfu cm^{-2} area). Among fifteen locations surveyed, the highest population phyllosphere bacterium obtained from samples, Mulleria (184.6 x 10^6cfu cm^{-2} flower area), followed by Natika (110.6 x 10^6cfu cm^{-2} flower area), Payyannur (109 x 10^6cfu cm^{-2} flower area) and it was found that phyllosphere

Fig. 4.4 Distribution of phyllosphere fungi on cowpea

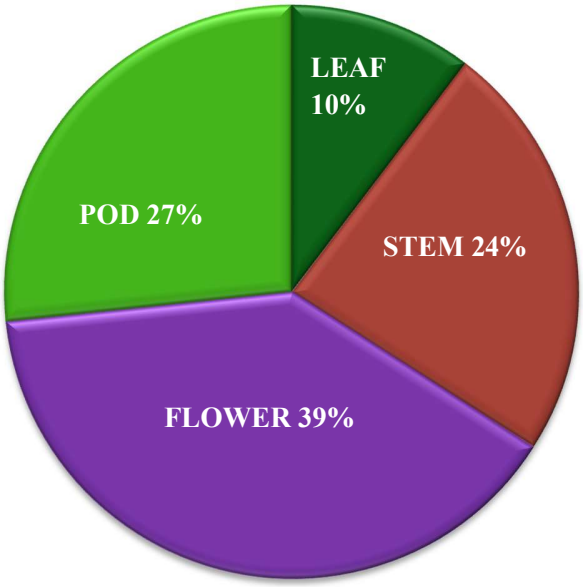
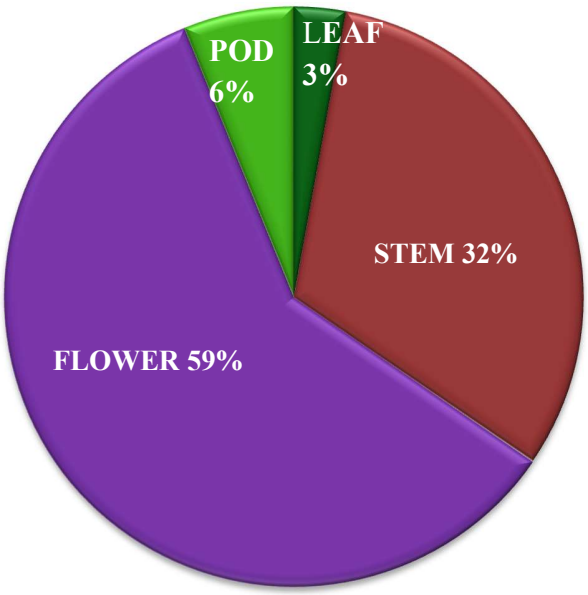


Fig. 4.5 Distribution of phyllosphere bacteria on cowpea



bacterium is abundant in cowpea plants, especially in flowers and ranks first among the all other phyllosphere microflora (Fig 4.5).

The results of isolation and enumeration of phyllsophere microbes showed thatcowpea flowers harbour plenty of fungal and bacterial communities. Flower organs are known to differ in epidermal cell structure and topography as well as chemically, through local exudations of sugars (nectar, stigmatic exudations, pollen exudations) (Heslop - Harrison,1985) and volatiles (Junker *et al*., 2011). As we know, flowers are the reproductive structures of the plant, its ephemerality and exquisite anatomy, flowers provide unique habitats to microorganisms (Aleklett *et al*., 2014). In this study, it was found that bacterial population is very abundant in cowpea phyllosphere. Bacteria are the most abundant inhabitants of the phyllosphere and epiphytic bacterial populations differ sharply in size among and within plants of the same species (Hirano and Upper, 1989; Lindow and Brandl, 2003). Similarly, fungus population in the phyllsphere was found high compared to fluorescent pseudomonads and yeasts and they are the important component of microbial communities and play major roles in ecosystem functions (Yao *et al*., 2019).

4.3.3 Population of phyllosphere fluorescent pseudomonads on cowpea

It was found that the mean population of phyllosphere fluorescent pseudomonads were more in cowpea pods (1.77 x 10^6cfu cm^{-2} area), followed by stems (1.12 x 10^6cfu cm^{-2} area), flowers (0.16 x 10^6cfu cm^{-2} area) and leaves (1.07 x 10^6cfu cm^{-2} area). Among fifteen locations surveyed, the highest population of phyllosphere bacterium obtained from samples, Mulleria (8.27 x 10^6cfu cm^{-2} pod area), followed by Mudicode (4.07x10^6 $cfucm^{-2}$ pod area), Payyannur (1.93x$10^6$$cfucm^{-2}$flower area) and it was found that phyllosphere fluorescent pseudomonads is less abundant in cowpea plants, and it ranks third among total population of all other phyllosphere microflora (Fig 4.6).

4.3.4 Population of phyllosphere yeasts on cowpea

It was found that, the mean population of phyllosphere yeast's were more in cowpea pods (1.32 x 10^4cfu cm^{-2} area), followed by flowers (1.21 x 10^4cfu cm^{-2} area), leaves (1.09 x 10^4cfu cm^{-2} area) andstems (1.03 x 10^4cfu cm^{-2} area). Among fifteen locations surveyed, the highest population phyllosphere yeasts obtained from samples,

Natika (4.54 x 10^4cfu cm^{-2} pod area), followed by Natika (3.04 x10^4cfu cm^{-2} flower area), Mulleria (1.9 x 10^4cfu cm^{-2} flower area) and it was found that phyllosphere yeast population is less abundant in cowpea plants, compared to other microbes and it ranks fourth among total population of all other phyllosphere microflora (Fig 4.7).

Table 4.6 Population of phyllosphere fungi on cowpea at different locations

District	Location	Fungi (x 10^2cfu cm^{-2} area)			
		Leaf	Stem	Flower	Pod
Thrissur	Chirakkekode1	3.95 *(0.58)^{e}	4.45 (0.65)^{f}	4.80 (0.68)^{f}	1.74 (0.24)^{g}
	Vellanikkara (Dept. of Vegetable science)	6.10 (0.78)^{b}	6.60 (0.82)^{d}	1.21 (0.04)^{l}	1.2 (0.03)^{kl}
	Kallingalpadam	4.50 (0.71)^{c}	17.06 (1.23)^{b}	12.21(1.08)^{c}	3.00 (0.49)^{d}
	Madannur	1.44 (0.18)^{h}	1.07 (0.03)^{i}	1.70 (0.23)^{k}	2.79 (0.44)^{e}
	Pandallur	2.41 (0.39)^{f}	1.05 (0.02)^{i}	2.80 (0.45)^{h}	1.08 (0.03)^{kl}
	Mala	1.83 (0.28)^{g}	1.19 (0.07)^{i}	1.00 (0.00)^{m}	1.10 (0.01)^{l}
	Madakkathara (KVK)	1.28 (0.11)^{i}	1.74 (0.24)^{h}	5.50 (0.75)^{e}	1.4 (0.15)^{i}
	Mannuthy	1.06 (0.02)^{j}	15.78 (1.19)^{b}	3.96 (0.59)^{g}	1.27 (0.10)^{j}
	Mudicode	2.30 (0.35)^{f}	2.28 (0.27)^{h}	9.28 (1.01)^{d}	1.58 (0.19)^{h}
	Chirakkekode 2	4.18 (0.62)^{de}	5.70 (0.75)^{de}	2.20 (0.33)^{j}	66.1 (1.82)^{a}
	Kazhimbram	1.61 (0.21)^{h}	7.30 (0.91)^{c}	3.80 (0.57)^{g}	4.02 (0.60)^{c}
	Natika	6.54 (0.81)^{b}	22.02 (1.34)^{a}	65.50 (1.81)^{a}	17.06 (1.23)^{b}
Malappuram	Manjeri	4.65 (0.67)^{cd}	1.07 (0.03)^{i}	2.42 (0.38)^{i}	1.12 (0.05)^{k}
Kannur	Payyannur	1.86 (0.27)^{g}	1.07 (0.73)^{e}	16.4 (1.21)^{b}	1.58 (0.20)^{h}
Kasaragod	Mulleria	6.87 (0.89)^{a}	5.40 (0.47)^{g}	16.45(1.21)^{b}	1.98 (0.30)f
Mean		3.37	6.44	10.00	7.11
CD (0.05)		0.055	0.064	0.021	0.032

* Values given in parenthesis are logarithmic transformed values with same super script are not significantly different

Fig. 4.6 Distribution of phyllosphere fluorescent pseudomonas on cowpea

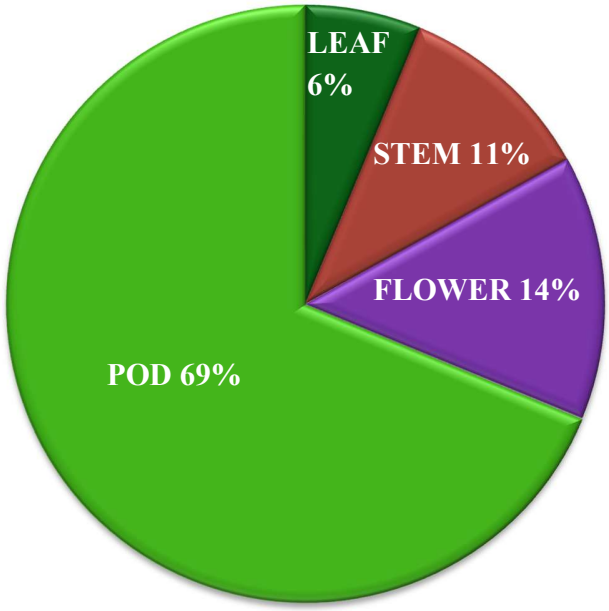


Fig. 4.7 Distribution of phyllosphere yeasts on cowpea

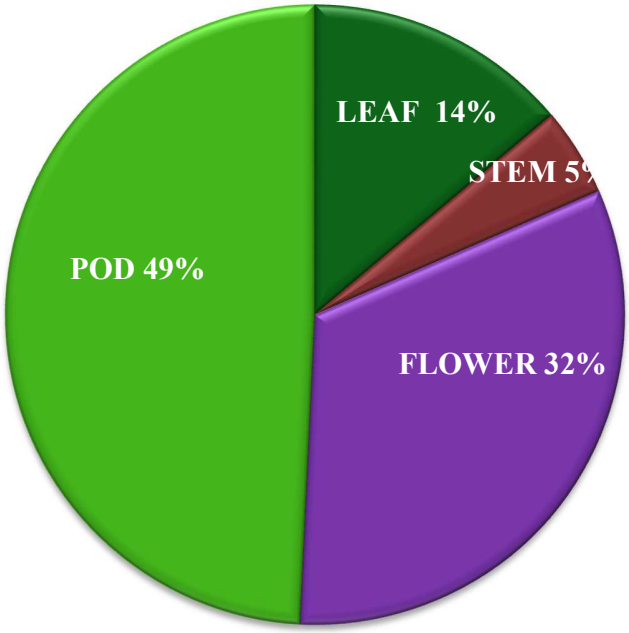


Table 4.7 Population of phyllosphere bacteria on cowpea at different locations

District	Location	Bacteria (x 10^6cfu cm^{-2} area)			
		Leaf	Stem	Flower	Pod
Thrissur	Chirakkekode1	1.60 *(0.20)^{h}	10.08 (1.01)^{d}	3.59 (0.55)^{j}	4.2 (0.62)^{e}
	Vellanikkara (Dept. of Vegetable science)	1.10 (0.06)^{l}	1.21 (0.08)^{m}	1.00 (0.00)^{m}	1.00 (0.00)^{k}
	Kallingalpadam	5.86 (0.79)^{b}	2.88 (0.46)^{i}	17.36 (1.23)^{f}	1.19 (0.07)^{i}
	Madannur	1.32 (0.13)^{j}	19.32 (1.28)^{b}	16.70 (1.22)^{g}	1.25 (0.09)^{h}
	Pandallur	2.61 (0.42)^{e}	1.49 (0.17)^{l}	1.21 (0.08)^{l}	1.20 (0.09)^{h}
	Mala	1.13 (0.05)^{l}	1.09 (0.02)^{n}	1.00 (0.00)^{m}	1.00 (0.00)^{k}
	Madakkathara (KVK)	1.69 (0.22)^{h}	1.20 (0.07)^{m}	13.7 (1.13)^{h}	6.50 (0.81)^{d}
	Mannuthy	1.54 (0.18)^{i}	16.07 (1.20)^{c}	108.4 (2.03)^{c}	1.55 (0.19)^{g}
	Mudicode	1.25 (0.09)^{k}	2.52 (0.39)^{j}	47.07 (1.67)^{d}	1.07 (0.03)^{j}
	Chirakkekode 2	2.21 (0.35)^{f}	3.25 (0.51)^{h}	3.25 (0.51)^{k}	1.22 (0.09)^{hi}
	Kazhimbram	1.97 (0.29)^{g}	6.40 (0.80)^{e}	6.62 (0.82)^{i}	18.82 (1.27)^{b}
	Natika	6.06 (0.79)^{a}	4.84 (0.68)^{e}	110.6 (2.04)^{b}	2.63 (0.42)^{f}
Malappuram	Manjeri	4.38 (0.63)^{c}	3.80 (0.58)^{f}	19.09 (1.28)^{e}	24.92 (1.39)^{a}
Kannur	Payyannur	3.48 (0.54)^{d}	2.10 (0.32)^{k}	109 (2.03)^{c}	6.46 (0.81)^{d}
Kasaragod	Mulleria	4.50 (0.65)^{c}	28.45 (1.45)^{a}	184.6 (2.26) ^{a}	7.58 (0.87)^{c}
Mean		3.04	23.42	42.94	5.37
CD (0.05)		0.018	0.016	0.003	0.012

* Values given in parenthesis are logarithmic transformed values with same super script are not significantly different

Table 4.8 Population of phyllosphere fluorescent pseudomonads on cowpea at different locations

District	Location	Fluorescent pseudomonas (x 10^6cfu cm^{-2} area)			
		Leaf	**Stem**	**Flower**	**Pod**
Thrissur	Chirakkekode1	0.05 *(0.02)	0.00 (0.00)	0.00 (0.00)	0.08 (0.03)
	Vellanikkara (Dept. of Vegetable science)	0.10 (0.04)	0.21 (0.08)	0.00 (0.00)	0.00 (0.00)
	Kallingalpadam	0.00 (0.00)	0.47 (0.16)	0.00 (0.00)	0.42 (0.15)
	Madannur	0.11 (0.04)	0.00 (0.00)	0.17 (0.06)	3.07 (0.61)
	Pandallur	0.00 (0.00)	0.00 (0.00)	0.16 (0.06)	0.00 (0.00)
	Mala	0.13(0.05)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	Madakkathara (KVK)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	Mannuthy	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	Mudicode	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	Chirakkekode 2	0.10 (0.04)	0.04 (0.017)	0.29 (0.11)	0.00 (0.00)
	Kazhimbram	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)	0.00 (0.00)
	Natika	0.00 (0.00)	0.25 (0.09)	0.24 (0.09)	0.00 (0.00)
Malappuram	Manjeri	0.34 (0.12)	0.28 (0.10)	0.00 (0.00)	0.00 (0.00)
Kannur	Payyannur	0.16 (0.06)	0.07 (0.02)	0.93 (0.28)	0.83 (0.26)
Kasaragod	Mulleria	0.11 (0.04)	0.52 (0.18)	0.97 (0.29)	7.27 (0.91)
Mean		0.07	0.12	0.16	0.77
CD (0.05)		Non significant			

* Values given in parenthesis are logarithmic transformed values with same super script are not significantly different

Table 4.9 Population of phyllosphere yeasts on cowpea at different locations

District	Location	Yeasts (x 10^4cfu cm^{-2} area)			
		Leaf	**Stem**	**Flower**	**Pod**
Thrissur	Chirakkekode1	0.00 *(0.00)	0.10 (0.03)^{ab}	0.17 (0.04)^{c}	0.00 (0.00)
	Vellanikkara (Dept. of Vegetable science)	0.00 (0.00)	0.00 (0.00)^{b}	0.00 (0.00)^{f}	0.00 (0.00)
	Kallingalpadam	0.06 (0.02)	0.00 (0.00)^{b}	0.00 (0.00)^{f}	0.00 (0.00)
	Madannur	0.00 (0.00)	0.00 (0.00)^{b}	0.00 (0.00)^{f}	0.38 (0.14)
	Pandallur	0.00 (0.00)	0.00 (0.00)^{b}	0.00 (0.00)^{f}	0.00 (0.00)
	Mala	0.00 (0.00)	0.00 (0.00)^{b}	0.00 (0.00)^{f}	0.00 (0.00)
	Madakkathara (KVK)	0.00 (0.00)	0.00 (0.00)^{b}	0.00 (0.00)^{f}	0.00 (0.00)
	Mannuthy	0.03 (0.01)	0.00 (0.00)^{b}	0.13 (0.04)^{e}	0.00 (0.00)
	Mudicode	0.14 (0.05)	0.00 (0.00)^{b}	0.14 (0.08)^{d}	0.00 (0.00)
	Chirakkekode 2	0.00 (0.00)	0.00 (0.00)^{b}	0.00 (0.00)^{f}	0.00 (0.00)
	Kazhimbram	0.00 (0.00)	0.00 (0.00)^{ab}	0.00 (0.00)^{f}	0.00 (0.00)
	Natika	0.22 (0.08)	0.34 (0.04)^{a}	2.04 (0.48)^{a}	0.36 (0.05)
Malappuram	Manjeri	0.00 (0.00)	0.00 (0.00)^{b}	0.00 (0.00)^{f}	0.00 (0.00)
Kannur	Payyannur	0.00 (0.00)	0.00 (0.00)^{b}	0.00 (0.00)^{f}	0.00 (0.00)
Kasaragod	Mulleria	0.28 (0.10)	0.13 (0.03)^{b}	0.73 (0.23)^{b}	0.9 (0.27)
Mean		0.04	0.03	0.32	0.21
CD (0.05)		NS	S (0.048)	S (0.00)	NS

S – significant , NS – Non significant

* Values given in parenthesis are logarithmic transformed values with same super script are not significantly different

Present study revealed that the population of fluorescent pseudomonads and yeasts are abundant in cowpea pods compared to other parts. However, we know that microbial load is more on flowers and development of pods from flowers obiviously maybe the reason for that. From the data, it was found that the weather parameters favours the growth of phyllosphere microbes. Plant exudates which contain simple sugars, organic acids and other easily utilized compounds which are the main nutrient source especially for yeasts (Jeyashri *et al*., 2019). Samples collected in summer months (March and April) yielded

highest population of phyllsophere fungi and bacteria compared to other season. It may be because of the humidity was slightly high (68 to 78 per cent) while in case of bacteria, highest population was found during the period of the late winter to summer (January, February, March). But in some locations low relative humidity affected the population of bacteria. The population of fluorescent pseudomonads and yeasts were much lower compared to fungi, bacteria and also low humidity affected their population in plants.

4.4 *IN VITRO* ANTAGONISTIC EFFECT OF PHYLLOSPHERE ANTAGONISTS TOWARDS THE PATHOGEN

4.4.1 Preliminary screening of phyllosphere microorganisms

Cowpea samples collected from various locations yielded 183 isolates, which includes 86 bacteria, 85 fungi and seven fluorescent pseudomonads and seven yeasts. They were subjected to preliminary screening in order to test the antagonistic property towards the pathogen. Out of the 183 isolates 142 did not exhibit any antagonism towards the pathogen, whereas, 41 found to be antagonistic in varying degrees. These isolates included 15 bacteria, 25 fungi and one yeast.

4.4.2 *In vitro* evaluation of antagonistic phyllosphere isolates

Preliminary screening revealed that 41 isolates are found exerting antagonism against the pathogen and they are subjected to further evaluation by dual culture method. Most promising and efficient isolates were screened out from dual culture method (Plate 4.14). Data on per cent inhibition of *C. siamense* by the phyllosphere isolates and their cataloguing were given in Table 4.10. It was revealed that, the extent of inhibition varied among the isolates, with the maximum of 95.5 per cent of inhibition by the isolate KVKSF1 and some of the fungal isolates showed good inhibition of pathogen, but they are coming under the genera *Aspergillus* from cultural and morphological characterization, since they produce aflatoxins and they are not selected for further studies. Hence best five non pathogenic isolates were selected and they varied from 76-90 per cent inhibition viz. KVKSF2 and KPCSB1 (with 90 per cent inhibition) followed by MUCSB1, CKDSF1and NKCSY1 with 82.2, 80, and 72.2 per cent inhibition respectively (Plate 4.10). Of the remaining ten isolates showed inhibition between 70-80

per cent, nine isolates showed inhibition between 60-70 per cent and 11 isolates showed inhibition between 50 – 60 per cent and rest of the isolates showed only 40-50 per cent of inhibition.

Table.4.10 Per cent inhibtionof *C. siamense* by antagonistic phyllosphere isolates

Sl no.	Isolate	District	Location	Part of the plant used	Type of organism	Per cent inhibition
1.	KVKSF1	Thrissur	Madakkathara(KVK)	Stem	Fungus	95.5
2.	KVKSF2	Thrissur	Madakkathara (KVK)	Stem	Fungus	90
3.	CKDSF1	Thrissur	Chirakkekode (CKD)	Stem	Fungus	84
4.	MLLF1	Thrissur	Mala (ML)	Leaf	Fungus	82
5.	MKDPF1	Thrissur	Mudicode (MKD)	Pod	Fungus	78.11
6.	PYRFF1	Kannur	Payyanuur (PYR)	Flower	Fungus	77.7
7.	MKDSF1	Thrissur	Mudicode (MKD)	Stem	Fungus	76
8.	KMLF1	Thrissur	Kazhimbram (KM)	Leaf	Fungus	74.5
9.	MTYLF1	Thrissur	Mannuthy (MTY)	Leaf	Fungus	73.3
10.	KMFF1	Thrissur	Kazhimbram (KM)	Flower	Fungus	73
11.	KMLF2	Thrissur	Kazhimbram (KM)	Leaf	Fungus	71
12.	NKCSF1	Thrissur	Natika (NK)	Stem	Fungus	68.1
13.	MKDSF2	Thrissur	Mudicode (MKD)	Stem	Fungus	68.02
14.	PYRPF1	Kannur	Payyannur (PYR)	Pod	Fungus	64
15.	NKCSF2	Thrissur	Natika (NK)	Stem	Fungus	61.8
16.	KPCSF1	Thrissur	Kallingalpadam (KP)	Stem	Fungus	55.5
17.	MUCSF1	Kasaragod	Muleria (MU)	Stem	Fungus	55.2
18.	MDCFF1	Thrissur	Madannur (MD)	Flower	Fungus	53.3
19.	PLRPF1	Thrissur	Panthallur (PLR)	Pod	Fungus	51

20.	PYRLF1	Kannur	Payyannur (PYR)	Leaf	Fungus	50.4
21.	MJCLF1	Malappuram	Manjeri (MJ)	Leaf	Fungus	50.02
22.	PYRPF2	Kannur	Payyannur (PYR)	Pod	Fungus	48.8
23.	KPCSF2	Thrissur	Kallingalpadam (KP)	Stem	Fungus	48.8
24.	NKCSF3	Thrissur	Natika (NK)	Stem	Fungus	45.4
25.	MTYLF2	Thrissur	Mannuthy (MTY)	Leaf	Fungus	44.11
26.	KPCSB1	Thrissur	Kanllingalpadam (KP)	Stem	Bacterium	90
27.	MUCSB1	Kasaragod	Mulleria (MU)	Stem	Bacterium	82.2
28.	MKDLB1	Thrissur	Mudicode (MKD)	Leaf	Bacterium	73
29.	PLRSB1	Thrissur	Panthallur (PLR)	Stem	Bacterium	72
30.	MKDLB2	Thrissur	Mudicode (MKD)	Leaf	Bacterium	68.8
31	NKCLB1	Thrissur	Natika (NK)	Leaf	Bacterium	65.83
32.	MTYFB1	Thrissur	Mannuthy (MTY)	Flower	Bacterium	63.8
33.	PYRFB1	Kannur	Payyannur (PYR)	Flower	Bacterium	62.2
34.	PYRLB1	Kannur	Payyannur (PYR)	Leaf	Bacterium	61.04
35.	KVKSB1	Thrissur	Madakkathara(KVK)	Stem	Bacterium	55.2
36.	CKD2PB1	Thrissur	Chirakkekode (CKD)	Pod	Bacterium	55
37.	PLRSFP1	Thrissur	Panthallur (PLR)	Stem	FP	54.8
38.	MKDSB1	Thrissur	Mudicode (MKD)	Stem	Bacterium	54.4
39.	KPCLB1	Thrissur	Kallingalpadam (KP)	Leaf	Bacterium	53.8
40	MUCLFP1	Kasaragod	Mulleria (MU)	Leaf	FP	44.08
41.	NKCSY1	Thrissur	Natika (NK)	Stem	Yeast	76.6

CL – Cowpea leaf, CS – Cowpea stem, CF - Cowpea flower, CP – Cowpea pod, L –Leaf, S- Stem, F- Flower, P- Pod, F – Fungus, B- Bacteria, FP- Fluorescent pseudomonads, Y-Yeast

Table 4.11Details of promising antagonistic phyllosphere isolates from cowpea

Sl no.	Isolate code	Location	Plant part used	Mean colony diameter (cm)	Per cent inhibition
1.	CKDSF1	Chirakkekode (CKD)	Stem	1.4 *(0.38) [b]	84 %
2.	KVKSF2	KVK (Madakkathara)	Stem	0.9 (0.27)[a]	90 %
3.	KPCSB1	Kallingalpadam (KP)	Stem	0.9 (0.27) [a]	90%
4.	MUCSB1	Mulleria (MU)	Stem	1.6 (0.41) [c]	82.2%
5.	NKCSY1	Natika (NK)	Stem	2.1 (0.49) [d]	76.6%
6.	Control	-	-	9.0 (1.00) [e]	0%
CD (0.01)				0.001	

* Values with same super script are not significantly different

From the study, it was found that, the antagonists of *Colletotrichum* causing anthracnose of cowpea is more abundant on cowpea stem and leaves (Fig 4.8). Surprisingly, it is known that, the anthracnose infection and symptoms are mostly seen on these parts (Onesirosan and Barker, 1971; Enyiukwu and Awurum, 2013; Satpathy and Beura, 2021). It is evident that there may be an existence of relationship between the host-pathogen- antagonists. Hence it proves the theory of co-evolution (Kurian, 2011; Thrall *et al*., 2012).

4.4.3 Phyllsophere antagonists selected for field evaluation

Since the main objective of this study was to identify the most potential phyllosphere microbes for management of cowpea anthracnose, antagonists were subjected to further evaluation to select five out of forty one antagonists. Thus, when tested individually by dual culture method, it is observed that, 36 isolates recorded more than 50 per cent of inhibition of the pathogen. Among this best five antagonists with highest

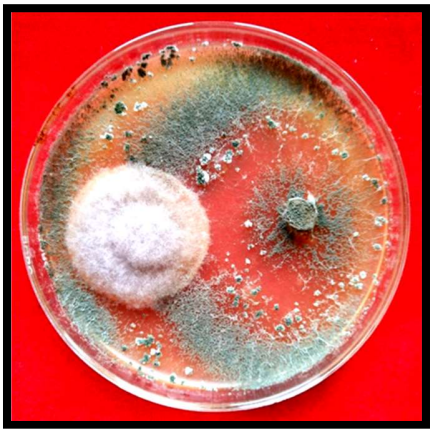
mycelial inhibition of the pathogen, including two fungi, two bacteria and one yeast isolate were selected for further evaluation.

It is well known that *in vitro* results on antagonistic effects do not necessarily translate directly to what occurs in natural field conditions. Nonetheless, *in vitro* studies and their results are particularly useful for identifying likely candidates for biocontrol and for predicting the mode of action/ antagonism by which they reduce pathogen damage (Mejia *et al*., 2008). Several studies have shown that the interaction between plants and certain phyllosphere microorganisms was associated with beneficial effects such as biocontrol of fungal pathogens and plant growth promotion (Zhang *et al*., 2015; Shang *et al*., 2020; Annadurai *et al*., 2020). On the other hand many phyllopshere microorganisms have failed to show any beneficial effects on the inoculated host plant (Williams *et al*., 2014). Moreover, selection and identification of antagonists with growth promoting and disease suppressive efficacy through *in vitro* and *in vivo* assays are crucial for development of efficient biocontrol strategy before conducting field trials (Weller, 1988). In the preliminary screening, it was found that out of the 183 phyllopshere isolates collected from cowpea , 41 viz. about 25 per cent of the total, were able to exert antagonism in varying degrees while the remaining were neutral. Similar line of work has been carried out by Adebanjo and Bankole in 2004 , but in that study only cowpea phylloplane is focussed and inhibition of growth of the pathogen *C. lindemuthianum* with production of zones of inhibition was observed for *Aspergillus flavus*, *A. ochraceus*, *Penicillium aurantiogriseum*, *Bacillus subtilis*- BS21, *B. subtilis*- BS22 and *B. subtilis*- BS23.This study also shows that cowpea phyllopshere is playing a major role in inhabiting a huge number of beneficial microbes that are very effective against *Colletotrichum* fungus.

4.5 CHARACTERIZATION OF PHYLLOSPHERE ANTAGONISTS

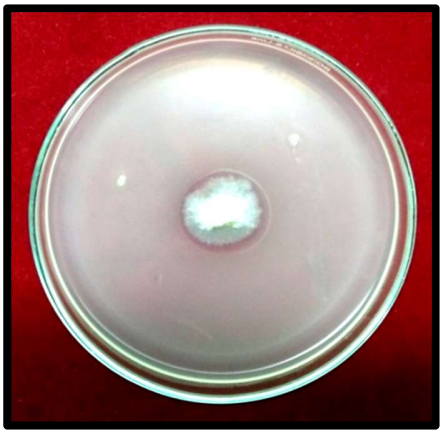
Best five phyllosphere antagonists were selected based on *in vitro* evaluation and purified. It includes two fungal isolates (CKDSF1, KVKSF2), two bacterial isolates (KPCSB1, MUCSB1) and one yeast isolate (NKCSY1). Cultural and morphological characterization were performed to aid in further identification.

Plate 4.10 Promising phyllosphere antagonists of *C. siamense*

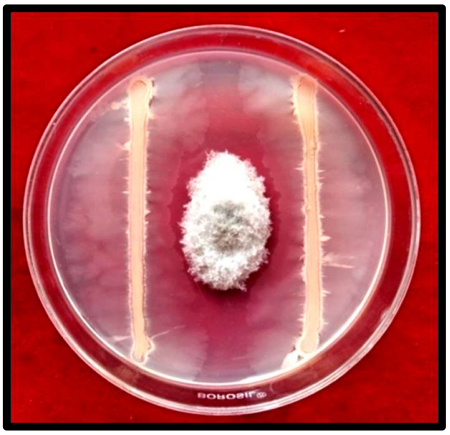


CKDSF1

KVKSF2



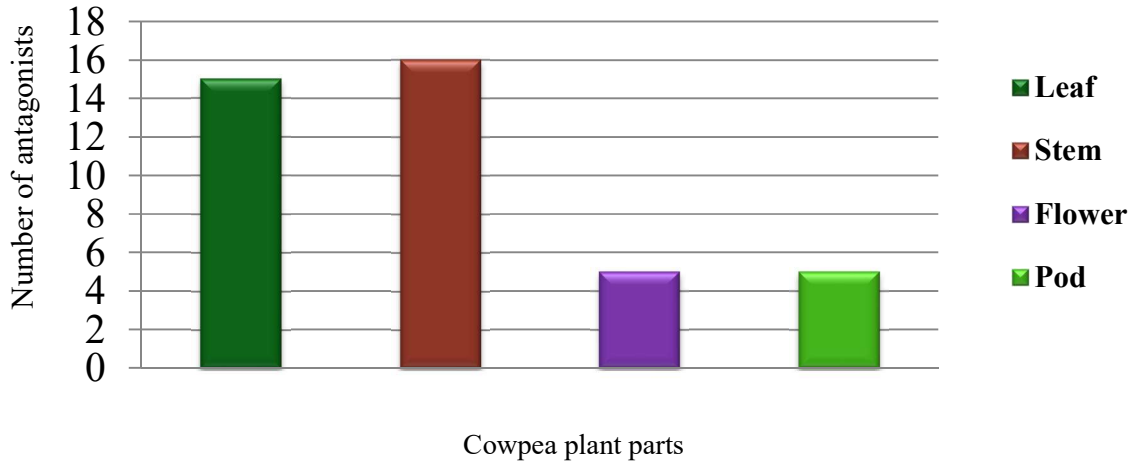
KPCSB1



MUCSB1

NKCSY1

Fig. 4.8 Distribution of promising phyllosphere antagonists on cowpea



4.5.1. Cultural and morphological characterization of fungal isolates

The cultural characters of the fungus *viz*. colour, texture, growth rate, growth pattern, fructifications, sporulation and pigmentation on the reverse side of the Petri dishes was studied. Thereafter, the morphological characters *viz*. colour and branching pattern of hyphae, hyphal and conidial septation, colour, shape and dimensions of spores and presence of fruiting bodies was studied (Table 4.12) (Plate 4.15).

Table 4.12 Cultural and morphological characterization of phyllosphere antagonistic fungi

Isolate	Cultural characters			Morphological characters		
	Colour	Texture	Days for complete growth	Shape of conidia	Size of conidia	Remarks
CKDSF1	Pale green myceliumand yellow to pale green on reverse side of Petri dish	Flat mycelium with small cottony pustules	3 days	Elllipsoidal to oblong	1.37-2.23µm	Conidiophores with Phialides
KVKSF2	Dark green to pale green myceliumand creamy colour on reverse side of Petri dish	Fluffy and flat growth radially	3 days	Elllipsoidal to oblong	1.23 - 1.59µm	Conidiophores with phialides

4.5.2.Cultural and morphological characterization of antagonistic phyllodphere bacteria and yeasts of cowpea

The cultural characters of the bacterial colonies were studied by growing on nutrient agar and the characters observed were the colour, pigmentation, texture and mucoidal nature of the developing colonies. The morphology of bacterial cells such as shape and size were observed under light microscope (400X) and with the aid of electron microscope (15000X) (Table 4.13).

4.5.2.1 Cultural characterization

For bacteria, colony growth was started within 21 hours of incubation in nutrient agar. Both the isolates (KPCSB1, MUCSB1) produced small to medium sized, circular to oval smooth and pale creamy colonies that were non mucoid in nature. The bacterial colonies were thick, convex and flat (Plate 4.16 A&B) and for yeast isolate (NKCSY1) (Plate 4.16 C) colony growth was started within 20 hours of incubation in nutrient agar. The isolate produced small to medium sized, circular, raised and milky white colonies that were non mucoid in nature.

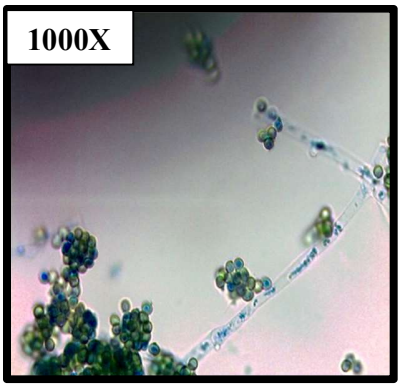
4.5.2.2 Characterization based on morphological characters

The morphology of bacterial cells was initially studied under light microscope with a magnification of 400 X and 1000 X and revealed that both the isolates *viz.* KPCSB1 and MUCSB1 were rod in shape. Short rods stained in dark violet were visible in Gram's staining reaction which confirmed that the isolates were gram positive. Further detailing of morphological characters were done using scanning electron microscope (Tescan Vega-3 LMU) from Central Instrumentation laboratory, College of Veterinary and Animal Sciences, Mannuthy. The electron microscopy analysis confirmed that the bacterial isolates (KPCSB1 and MUCSB1) were rod shaped. The isolate KPCSB1 was 0.66 to 1.12 µm in length and 0.37 to 0.40 µm breadth (Plate 4.21). The microscopic image was captured at a working distance of 4.99 mm and a SEM voltage of 10.0 kV which was fixed by trial and error method.The isolate MUCSB1 was 1.27 to 1.73µm in length and 0.82 to 0.97 µm breadth. The microscopic image was captured at a working distance of 10.01 mm and a SEM voltage of 10.0 kV which was fixed by trial and error method.

The morphology of yeast cells was initially studied under light microscope with a magnification of 400 X and 1000 X and revealed that the isolate NKCSY1 were oval in shape and presence of hypha and budding nature was observed. Further detailing of morphological characters were done using scanning electron microscope (Tescan Vega-3 LMU) from Central Instrumentation laboratory, College of Veterinary and Animal Sciences, Mannuthy. The electron microscopy analysis confirmed that the yeast isolate NKCSY1 was oval shaped and 4.14 to 5.64µm in length and 4.14 to 4.24 µm breadth. The

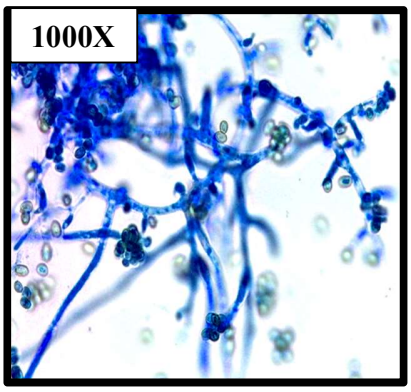
Plate 4.11 Cultural and morphological characterization of phyllosphere antagonistic fungi and yeast

CKDSF1



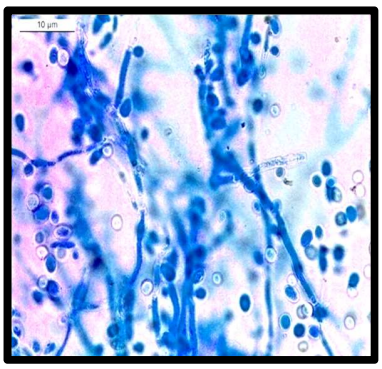
Conidia (1.37- 2.23µm)

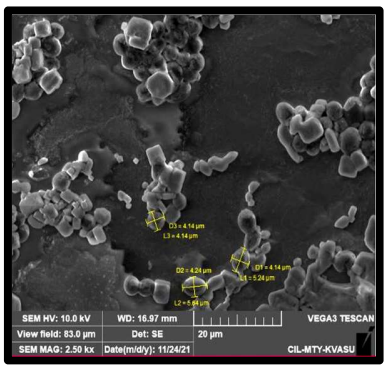
KVKSF2



Conidia (1.23 - 1.59µm)

NKCSY1





4.14 - 5.64 x 4.14 - 4.24 µm

microscopic image was captured at a working distance of 16.97 mm and a SEM voltage of 10.0 kV which was fixed by trial and error method.

Table 4.13 Cultural and morphological characterization of phyllosphere antagonistic bacteria and yeast

Cultural and morphological characters	KPCSB1	MUCSB1	NKCSY1
Colour	Creamy white	Creamy white	Milky white
Texture	Smooth	Smooth	21h
Mucoidal nature	Absent	Absent	Absent
Flat/ raised	Flat	Flat	Raised
Time taken for full growth	21h	21h	20h
Shape of colony	Small, Circular to ovoid	Small, Circular to ovoid	Small, Circular
Size of colony	0.66 -1.12 X 0.37- 0.40 µm	1.27- 1.73 X 0.82 -0.97 µm	4.14 - 5.64 X 4.14 - 4.24 µm
Remarks	Gran positive	Gram positive	Yeast like growth, budding nature

4.6 Molecular characterization of phyllosphere antagonists

For species level identification and confirmation of phyllosphere antagonists (CKDSF1, KVKSF2, KPCSB1, MUCSB1, NKCSY1), molecular characterization was done. The ITS regions of fungal and yeast DNA and 16S-rRNA regions of bacterial genomic DNA were PCR amplified using specific primers at Rajiv Gandhi Centre for Biotechnology (RGCB), Thiruvananthapuram. The sequences obtained were analysed using BLASTn search against NCBI nr database.

4.6.1. CKDSF1 (*Trichoderma longibrachiatum*)

The BLASTn analysis of ITS sequences of the phyllopshere fungal isolate CKDSF1 in NCBI nr database showed 99.25 per cent identity with *T. longibrachiatum* small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S

ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (MT052707.1) with 99 per cent query cover (Table 4.14). Along with this, the sequence showed 99.25 per cent similarity with the accessions of the same pathogen (MT052706.1, MN416777.1, MK849898.1).

4.6.2 KVKSF2 (*Trichoderma asperellem*)

The BLASTn analysis of ITS sequences of the phyllopshere fungal isolate KVKSF2 in NCBI nr database showed 98.16 per cent identity with *T. asperellem* small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence (MH398560.1) with 100 per cent query cover (Table 4.14). Along with this, the sequence showed 98.16 per cent similarity with the accessions of the same isolate (MF780711.1, MG675228.1, CP084949.1).

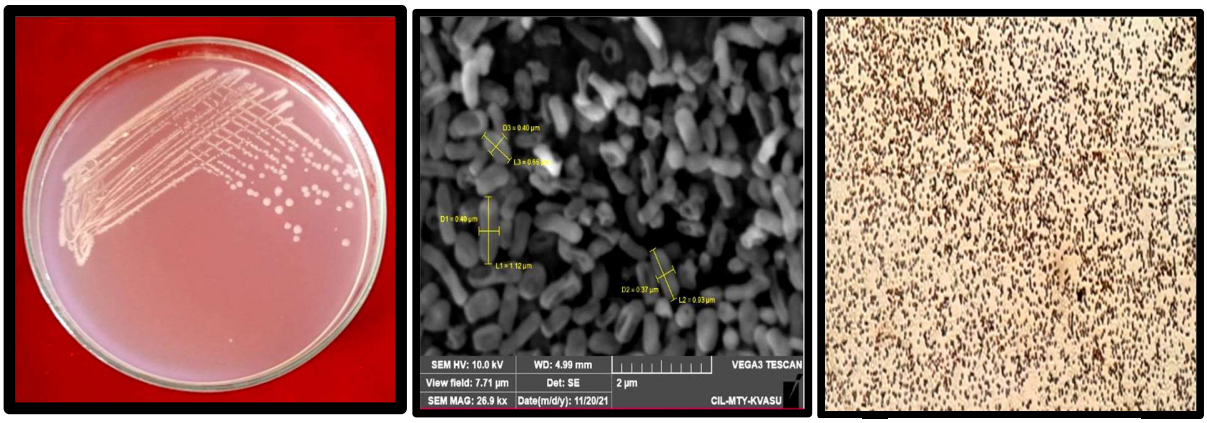
Table.4.14 *In silico* analysis of ITS sequencesantagonistic phyllosphere fungi

Isolate	Description	Max score (%)	e – value	Query coverage (%)	Identity percentage
CKDSF1	*Trcihoderma longibrachiatum*	965	0.0	99	99.25
KVKSF2	*Trichoderma asperellem*	758	0.0	100	98.16

The evolutionary relationship between CKDSF1 and other top hits obtained from BLASTn analysis in NCBI nr database revealed that CKDSF1 was more related to the accession of *T. viride* (MN807256.1) and distantly related to the accessions of the same isolate MK084475.1 and MK765011.1. The evolutionary relationship between *Trcihoderma asperellem* (KVKSF2) and other top hits obtained from BLASTn analysis in NCBI nr database revealed that KVKSF2 was more related to the accession of *T. asperellem* S1 26-R (MG675228.1) and distantly related to the accessions of the same isolate (MH398560.1) (Fig 4.9).

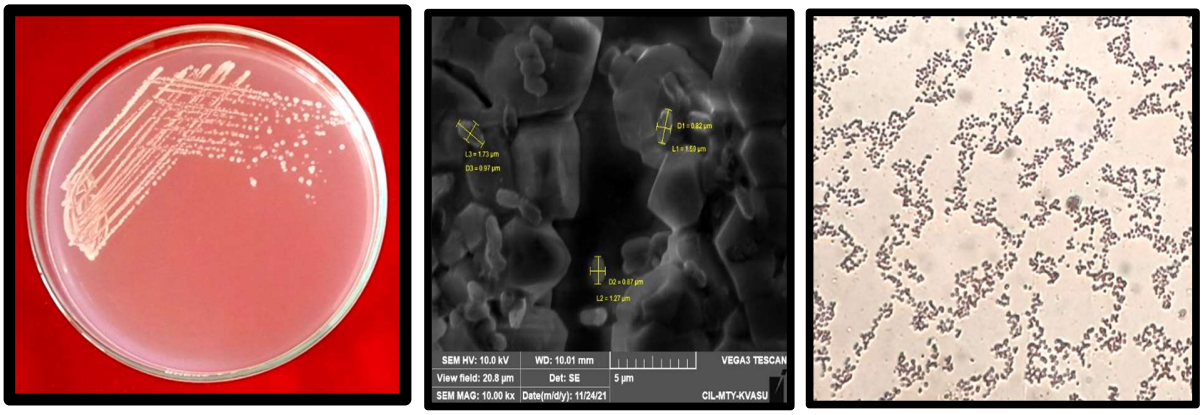
Plate 4.12 Cultural and morphological characterization of antagonistic bacteria

(A) KPCSB1



0.66 -1.12 x 0.37- 0.40 µm **Gram positive**

(B) MUCSB1



1.27- 1.73 x 0.82 -0.97 µm **Gram positive**

Fig 4.9 Neighbor-joining phylogenetic tree constructed in Mega X showing relationship between various species of the genus *Trichoderma*

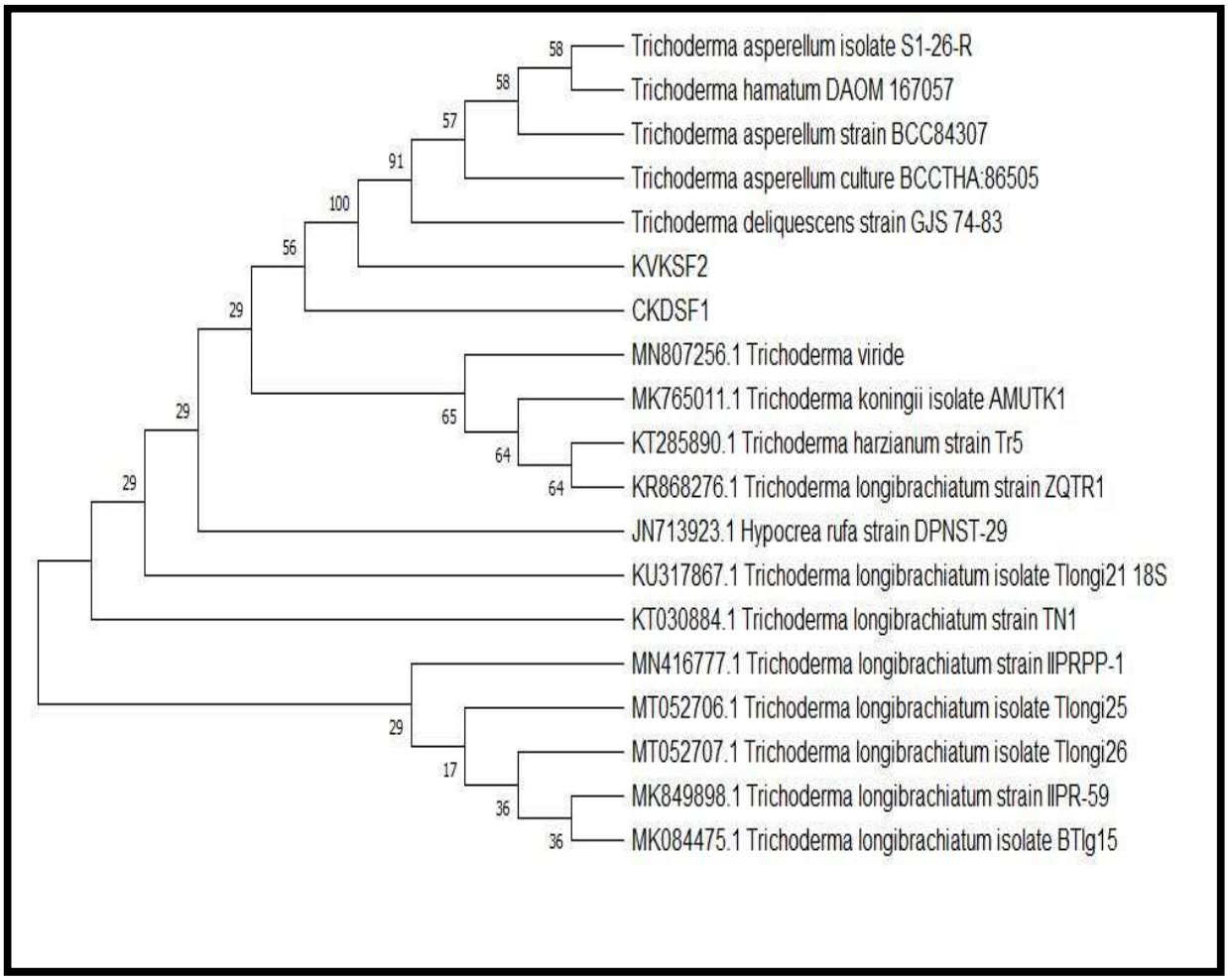
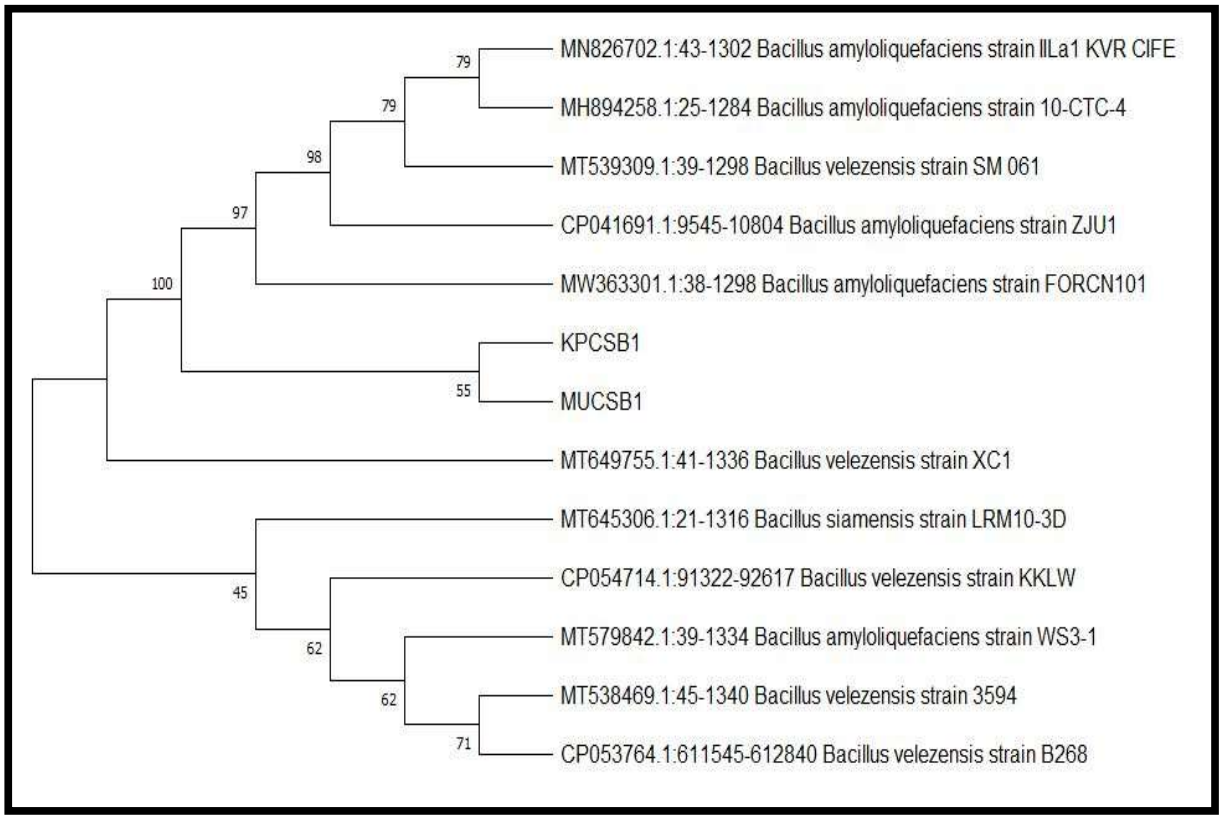


Fig 4.10 Neighbor-joining phylogenetic tree constructed in Mega X showing relationship between various species of the genus *Bacillus*



4.6.3 KPCSB1 (*Bacillus amyloliquefaciens*)

The BLASTn analysis of 16S rDNA sequencing of the phyllopshere bacterial isolate KPCSB1 in NCBI database showed 99.92 per cent identity with *B. amyloliquefaciens* strain FORCN101 16S ribosomal RNA gene, partial sequence (MW363301.1) with 100 per cent query cover (Table 4.15). Along with this, the sequence showed 99.92 per cent similarity with the accessions of the same isolate (MT539309.1, MN826702.1, MH894258.1).

4.6.4 MUCSB1 (*Bacillus velezensis*)

The BLASTn analysis of 16S rDNA sequencing of the phyllopshere bacterial isolate MUCSB1 in NCBI database showed 99.84 per cent identity with *B. velezensis* strain XC1 16S ribosomal RNA gene, partial sequence (MT649755.1) with 100 per cent query cover (Table 4.15). Along with this, the sequence showed 99.84 per cent similarity with the accessions of the same isolate (MT645306.1, MT579842.1, CP054714.1, CP053764.1).

Table.4.15 *In silico* analysis of antagonistic phyllosphere bacteria

Isolate	Description	Max score (%)	e – value	Query coverage (%)	Identity percentage
KPCSB1	*Bacllius amyloliquefaciens*	2324	0.0	100	99.92
MUCSB1	*Bacillus velezensis*	2329	0.0	99	99.84

The evolutionary relationship between KPCSB1 and other top hits obtained from BLASTn analysis in NCBI nr database revealed that KPCSB1 was more related to the accession *B. amyloliquefaciens* strain FORCN101(MW363301.1) and distantly related to the accessions of the same isolate (MT539309.1).The evolutionary relationship between MUCSB1 and other top hits obtained from BLASTn analysis in NCBI nr database revealed that MUCSB1 was more related to the accession *B. velezensis* strain B268 (CP053764.1) and distantly related to the accessions of the same isolate (MT538469.1) (Fig 4.10).

4.6.5 Isolate NKCSY1

The cultural and morphological characterization of yeast isolate NKCSY1 identified as *Candida* sp. The BLASTn analysis of ITS sequences of isolate NKCSY1 in NCBI nr database showed 99.40 per cent identity with *Candida tropicalis* strain MYA-3404 chromosome R, (CP047875.1) with 98 per cent query cover (Table 4.16). The evolutionary relationship between *C. tropicalis* (NKCSY1) and other top hits obtained from BLASTn analysis in NCBI nr database revealed that NKCSY1 was more related to *C. parapsilopsis* (XR005013740.1) the accession ofand distantly related to the accessions of the same isolate (HE681725.1) (Fig 4.11).

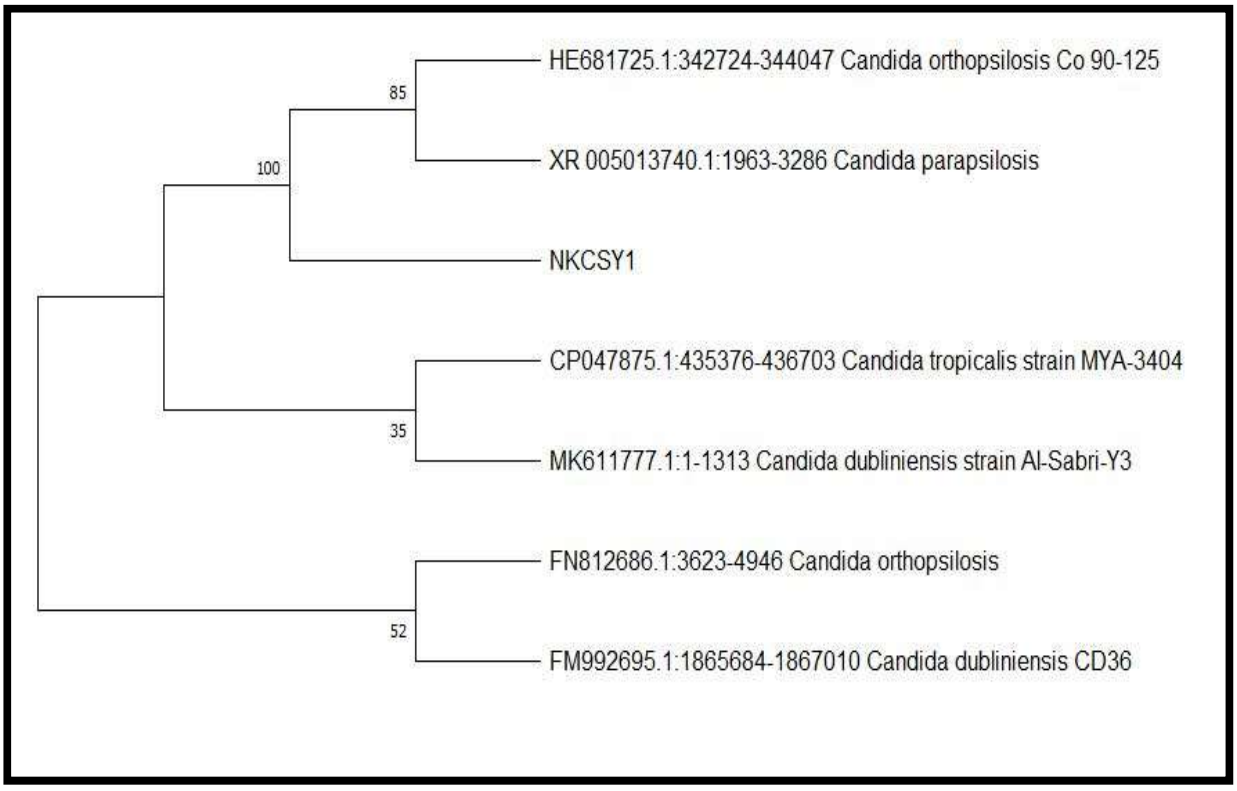
Table.4.16 *In silico* analysis ITS sequences of phyllosphere yeast

Isolate	Description	Max score (%)	e – value	Query coverage (%)	Identity percentage
NKCSY1	*Candida tropicalis*	2381	0.0	98	99.40

The species level identification of all the five potential antagonists were done based on molecular characterization.BLASTn analysis of CKDSF1 showed that the isolate is *Trichoderma longibrachiatum*and on phylogenetic anlaysis resulted the same. BLASTn analysis as well as phylogenetic tree analysis showed that the isolate KVKSF2 is *T. asperellem.* Based on neighbor-joining phylogenetic tree constructed in Mega X showing relationship between various species of the genus *Trichoderma,* it has been proved that both the isolates are different species. As we know, *Trichoderma* spp. are more efficient in managing plant pathogens. *T. asperellem* is an efficient mycoparasite of *C. gloeosporioides* (Quiroz *et al*., 2018) and C. *gloeosporioides* C62 causing tea anthracnose (Shang *et al*., 2020). In this study the phyllosphere isolates *T. longibrachiatum* and *T. asperellem* showed inhibitory action against the pathogen *C. siamense*.

The bacterial isolate, KPCSB1 was identified as *B. amyloliquefaciens*and MUCSB1 identified as *B. velezensis*. Both the species are classified under *B. subtilis* species complex (Fan *et al*.,2017; Rabbee *et al*., 2019) In this study both the phyllosphere bacterial isolates *B. amyloliquefaciens* and *B. velezensis* showed mycelial inhibition of the

Fig 4.11 Phylogenetic tree of *Candida* sp. (NKCSY1) by bootstrap method



pathogen *C. siamense* and this is an indication of release of diffusible metabolites by the bacterium (Esh *et al*., 2011). There are some other reports on *B. amyloliquefaciens* showing antagonism towards pathogens like *Cercospora beticola* (Esh *et al*., 2011), *C. gloeosporioides* (Mochizuki *et al*., 2012), *Blumeria graminis* f. sp. *tritici* (Hongfeng *et al*., 2014) and *Colletotrichum truncatum* (Gowtham *et al*., 2018). The phyllsopshere bacteria *B. velezensis* also showed antagonism towards *C. siamense.* There areother reports on antagonism by this bacteria against *Colletotrichum* (Jin *et al*., 2020; Kim *et al*., 2021; Choub *et al*., 2021) and various other pathogens (Jiang *et al*., 2018; Fan *et al*., 2018; Myo *et al*., 2021).BLASTn analysis of isolate NKCSY1 was identified as *Candida tropicalis* and phylogenetic analysis showed that the isolate is more similar to *C. parapsilopsis. Candida tropicalis* isolated from phyllosphere is known to inhibit the mycelial growth of the pathogen *C. siamense* and other species were reported such as, *C. gloeosporioides* (Sriram and Poornachanddra, 2013) *C. musae* (Zhimo *et al*., 2016). *C. parapsilopsis* is known to act as biocontrol agent againstaflatoxigenic *Aspergillus* species (Niknejad *et al*., 2012). All the five phyllosphere antagonists are well known beneficial microbes in plant disease management.

4.8 INDUCTION OF SYSTEMIC RESISTANCE IN COWPEA

The activity of various defense related enzymes was studied using spectral analysis at 0, 3, 5, 7 days interval. The results of the enzyme assays are presented below.

4.8.1 Induction of peroxidase (PO) by phyllosphere antagonists

Application of phyllosphere antagonists resulted in an increase in the activity of PO. The activity of PO as expressed by the change in absorbance ranged from control to before inoculation. Spectroscopic analysis of peroxidase activity against *C. siamense* was carried out and results are presented in (Table 4.17). Before challenge inoculation with pathogen, the treatment *B. velezensis* (T_4) showed highest peroxidase activity of 0.502 $min^{-1}g^{-1}$ and 0.281 $min^{-1}g^{-1}$ increase over the control, followed by *Candida tropicalis* (T_5). Inoculation of pathogen *C. siamense* resulted in PO activity over time and the treatment *T. longibrachiatum* (T_1) recorded highest PO activity of 0.564 $min^{-1}g^{-1}$ at three days after inoculation, followed by *C. tropicalis* (T_5) which

showed 0.561 $min^{-1}g^{-1}$. The lowest activity at 1DAI was observed in control (T_7), which recorded 0.234 $min^{-1}g^{-1}$. At five days after inoculation highest activity was recorded by T1 (0.791 $min^{-1}g^{-1}$) followed by the treatment *T. asperellem* (T_2) (0.778 $min^{-1}g^{-1}$). The expression of peroxidase activity elevated at seven days after inoculation of pathogen, where highest activity was noticed in plants treated with *B. amyloliquefaciens* (T_3) (1.47 $min^{-1}g^{-1}$), and was followed by *B. velezensis* (T_4)(0.986 $min^{-1}g^{-1}$). Control (T_6) recorded only 0.248 $min^{-1}g^{-1}$ (Fig 4.12).The activity increased from 0 DAI to 3, 5, 7 DAI in all the treatments.

The isolates *T. asperellem*, *T. longibrachiatum* and *Candida tropicalis* showed subsequent increase in PO activity at 0,3,5,7 DAI. But in the case of isolates of *B. amyloliquefaciens* and *B. velezensis* the activity was doubled 7DAI. In this study higher activity of peroxidase enzyme was recorded in plants treated with *B. amyloliquefaciens* and *B. velezensis*.

Table 4.17 Induction of peroxidase (PO) by phyllosphere antagonists

Treatment	0 DAI		3 DAI		5 DAI		7 DAI	
	PO activity $min^{-1}g^{-1}$ fresh tissue	**Per cent +/- over control**	**PO activity $min^{-1}g^{-1}$ fresh tissue**	**Per cent +/- over control**	**PO activity $min^{-1}g^{-1}$ fresh tissue**	**Per cent +/- over control**	**PO activity $min^{-1}g^{-1}$ *fresh* tissue**	**Per cent +/- over control**
T.longibrachiatum (T_1)	*0.482^{c}	+0.259	0.565^{a}	+0.072	0.791^{a}	+0.428	0.881^{d}	+0.636
T. asperellem (T_2)	0.197^{g}	-0.025	0.367^{f}	-0.125	0.778^{b}	+0.415	0.955^{c}	+0.708
B.amyloliquefaciens (T_3)	0.422^{d}	+0.201	0.438^{e}	-0.054	0.459^{e}	+0.096	1.470^{a}	+1.222
B. velezensis (T_4)	0.503^{a}	+0.281	0.521^{c}	+0.029	0.568^{d}	+0.205	0.987^{b}	+0.738
Candida trolicalis (T_5)	0.498^{b}	+0.277	0.562^{b}	+0.069	0.674^{c}	+0.311	0.776^{e}	+0.531
Control (T_6)	0.221^{e}	0	0.492^{d}	0	0.361^{f}	0	0.249^{g}	0
Absolute control (T_7)	0.218^{f}	-0.003	0.234^{g}	-0.258	0.246^{g}	-0.115	0.261^{f}	+0.015

* Values with same super script are not significantly different

Fig 4.12 Induction of peroxidase (PO) by phyllosphere antagonists

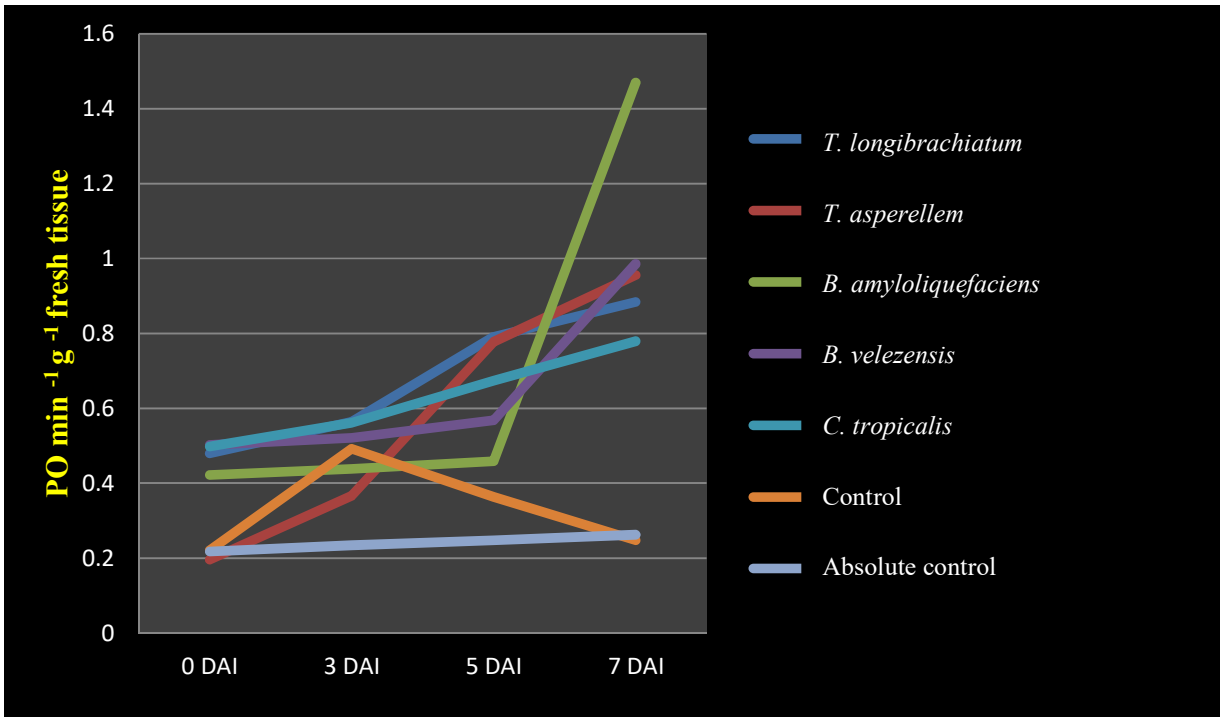
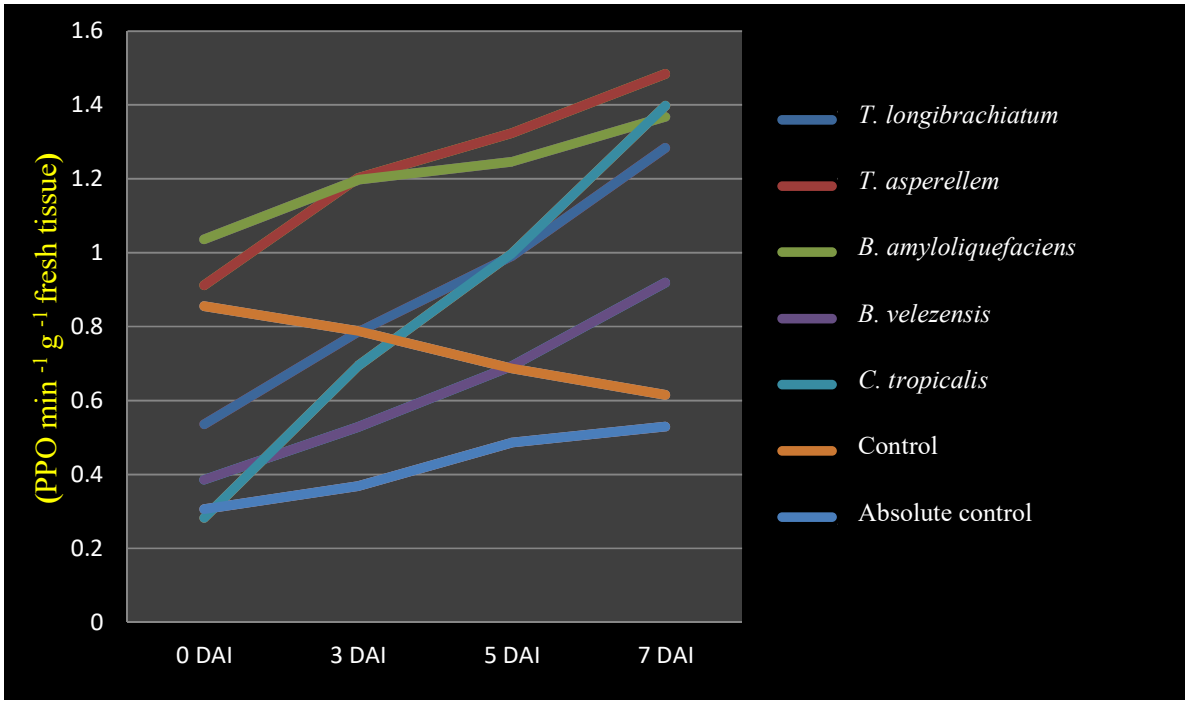


Fig 4.13 Induction of polyphenol oxidase (PPO) by phyllosphere antagonists



4.8.2 Induction of polyphenol oxidase (PPO) by phyllosphere antagonists

Application of phyllosphere isolates resulted in an increase in the activity of PPO (Table. 4.18). Before challenge inoculation, the highest activity (0.596 $min^{-1}g^{-1}$) was recorded by *B. amyloliquefaciens* (T_3), followed by Absolute control (T_7) (0.398 $min^{-1}g^{-1}$) and lowest activity was recorded by *T. longibrachiatum* (T_1) (0.258 $min^{-1}g^{-1}$). At three days after inoculation highest polyphenol oxidase activity was recorded by *B. amyloliquefaciens* (T_3) (0.612 $min^{-1}g^{-1}$), followed by *C. tropicalis* (T_5) (0.514 $min^{-1}g^{-1}$). At five days after inoculation, T_5 showed highest polyphenol oxidase activity (0.884 $min^{-1}g^{-1}$) and the same trend was followed seven days after inoculation (1.058 $min^{-1}g^{-1}$) and lowest activity was recorded by T_7 (absolute control) (0.464 $min^{-1}g^{-1}$) (Fig 4.13).However, there was subsequent increase in PPO activity by the isolates *T. asperellem*, *T. longibrachiatum*, *B. amyloliquefaciens*, *B. velezensis* and *C. tropicalis*. The phyllosphere antagonistic yeast isolate, *C. tropicalis* recorded highest activity, it was increased around 50 per cent at 7DAI. Higher activity of polyphenol oxidase (PPO) enzyme was recorded in plants treated with the yeast isolate, *C. tropicalis.*

4.8.3 Induction of phenylalanine ammonia lyase (PAL) by phyllosphere antagonists

Application of phyllosphere isolates resulted in an increase in the activity of PAL (Table. 4.19). The activity of PAL as expressed by the change in absorbance ranged from control to before inoculation. All the treatments synthesized more PAL compared to control. Before challenge inoculation the highest activity of enzyme was recorded by *B. amyloliquefaciens* (T_3) (1.036), followed by *T. asperellem* (T_2) (0.912) and lowest activity recorded by *C. tropicalis* (T_5) (0.282). At three days after inoculation highest activity shown by *T. asperellem* (T_2) (1.202) and least by absolute control (T_7) (0.368). At five days after inoculation of pathogen the highest enzyme activity was recorded by *T. asperellem* (T_2) (1.324), followed by *B. amyloliquefaciens* T_3 (1.246) and decreasing trend is observed in the control (T_6). At seven days after inoculation highest enzyme activity was recorded by *T. asperellem* T_2 (1.484), followed by *C. tropicalis* (T_5) (1.398) and lowest activity was recorded by T_7 (absolute control) (0.529) (Fig 4.14).

Table 4.18 Induction of polyphenol oxidase (PPO) by phyllosphere antagonists

Treatment	0 DAI		3 DAI		5 DAI		7 DAI	
	PPO activity $min^{-1}g^{-1}$ fresh tissue	Per cent +/-over control	PPO activity $min^{-1}g^{-1}$ fresh tissue	Per cent +/-over control	PPO activity $min^{-1}g^{-1}$ fresh tissue	Per cent +/-over control	PPO activity $min^{-1}g^{-1}$ fresh tissue	Per cent +/-over control
T.longibrachiatum (T_1)	*0.258^{e}	-0.03	0.398^{d}	+0.004	0.524^{d}	+0.105	0.712^{d}	+0.216
T. asperellem (T_2)	0.281^{c}	-0.007	0.369^{e}	-0.025	0.481^{e}	+0.062	0.587^{e}	+0.091
B.amyloliquefaciens (T_3)	0.596^{a}	+0.308	0.612^{a}	+0.218	0.684^{b}	+0.265	0.798^{b}	+0.302
B. velezensis (T_4)	0.276^{d}	-0.012	0.426^{c}	+0.032	0.594^{c}	+0.175	0.762^{c}	+0.266
Candida trolicalis (T_5)	0.259^{e}	-0.029	0.514^{b}	+0.12	0.884^{a}	+0.465	1.058^{a}	+0.562
Control (T_6)	0.288^{c}	0	0.394^{d}	0	0.419^{g}	0	0.496^{f}	0
Absolute control (T_7)	0.398^{b}	+0.11	0.364^{e}	-0.03	0.438^{f}	+0.019	0.464^{g}	-0.032

* Values with same super scriptarenot significantly different

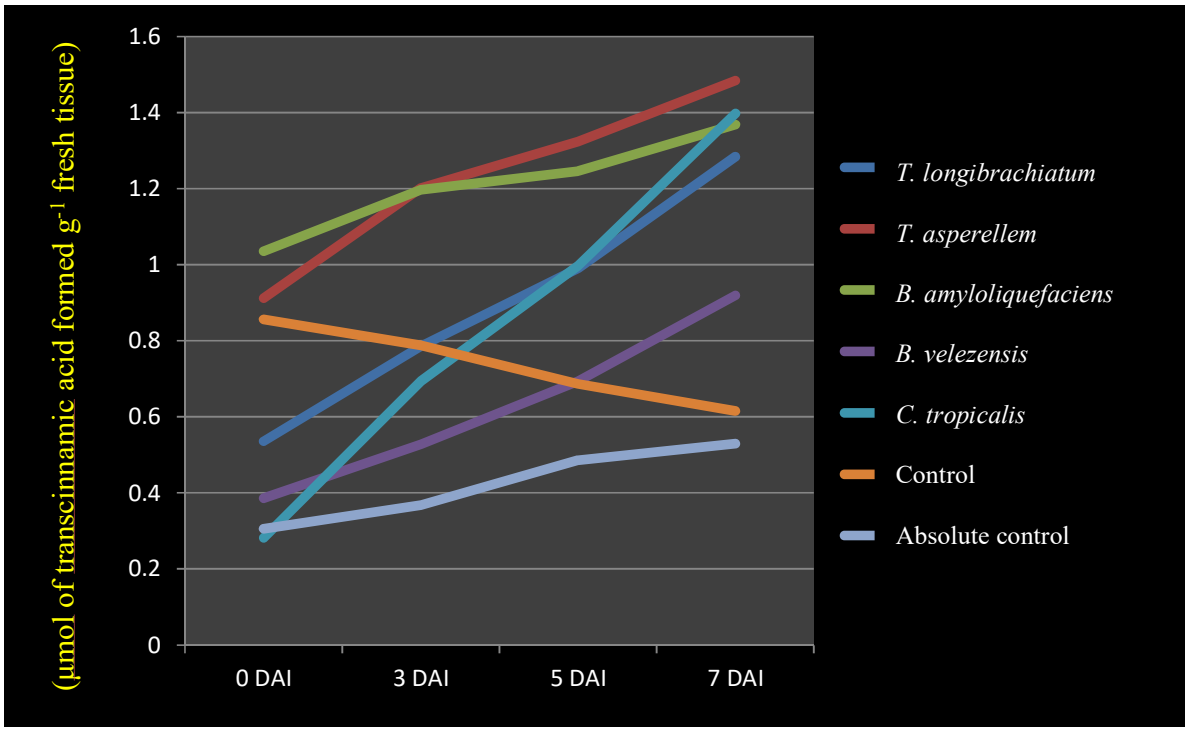
Table 4.19 Induction of phenlyalanine ammonia lyase (PAL) by phyllopshere antagonists

Treatment	0 DAI		3 DAI		5 DAI		7 DAI	
	PAL activity µmol of TCA formed g^{-1} fresh tissue	Per cent +/-over control	PAL activity µmol of TCA formed g^{-1} fresh tissue	Per cent +/-over control	PAL activity µmol of TCA formed g^{-1} fresh tissue	Per cent +/-over control	PAL activity µmol of TCA formed g^{-1} fresh tissue	Per cent +/-over control
T.longibrachiatum (T_1)	*0.536^{d}	-0.32	0.786^{b}	-0.002	0.991^{d}	+0.304	1.284^{d}	+0.669
T. asperellem (T_2)	0.912^{b}	+0.056	1.202^{a}	+0.414	1.324^{a}	+0.637	1.484^{a}	+0.869
B.amyloliquefaciens (T_3)	1.036^{a}	+0.18	1.197^{a}	+0.409	1.246^{b}	+0.559	1.368^{c}	+0.753
B. velezensis (T_4)	0.386^{e}	-0.47	0.528^{d}	-0.26	0.694^{e}	+0.007	0.919^{e}	+0.304
Candida trolicalis (T_5)	0.282^{g}	-0.574	0.694^{c}	-0.094	0.997^{c}	+0.31	1.398^{b}	+0.783
Control (T_6)	0.856^{c}	0	0.788^{b}	0	0.687^{f}	0	0.615^{f}	0
Absolute control (T_7)	0.306^{f}	-0.55	0.368^{e}	-0.42	0.486^{g}	-0.201	0.529^{g}	+0.086

* Values with same super script are not significantly different

* **TCA - Transcinnamic acid**

Fig 4.14 Induction of phenylalanine ammonia lyase (PAL) by phyllosphere antagonists



Apart from inhibiting the growth of pathogens phyllosphere antagonists have the ability to improve crop growth and enhancing plant resistance towards pathogen attack.*T.longibrachiatum* also plays an important role in induced system resistance in plants (Zhang *et al*., 2015) in addition to enhanced crop growth and vigour (Zhang *et al*., 2016; Montesinos *et al*., 2019). It is mainly achieved by production of indole 3 acetic acid and ACC deaminase (Zhang *et al*., 2019). Induced systemic resistance in plants has been reported by the application of *T. asperellem* (Shang *et al*., 2020).

Higher activity of defense related enzymes by *B. amyloliquefaciens* which induce systemic resistance reported by Gowtham *et al.* in 2018.*B. velezensis* induce systemic resistance in plants by production of 'acetoin' which further helps in production of H_2O_2 and thus aid in improving activity of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase enzymes (Peng *et al*., 2019; Rabbee *et al*., 2019; Chen *et al*., 2020).

4.9 EFFECT OF SECONDARY METABOLITES OF THE PHYLLOPSHERE ANTAGONISTS ON *Colletotrichum siamense*

Complete inhibition of the pathogen was observed on the medium amended with culture filtrate of the phyllosphere isolates indicating the inhibitor effect of the secondary metabolites (Table.4.20) (Plate 4.17).

Table 4.20 Effect of secondary metabolites of the phyllopshere antagonists on *C. siamense*

Sl no.	Phyllosphere isolate	Inhibition percentage
1.	*T. longibrachiatum*	100* (89.32[a])
2.	*T. asperellem*	100 **(89.32[a])
3.	*B. amyloliquefaciens*	98.8 (83.71[b])
4.	*B. velezensis*	98 (81.87[c])
5.	*Candida tropicalis*	86.6 (68.52[d])
6.	Control	0 (0.00[e])
CD (0.05)		S (0.01)

* Values in the parentheses are angular transformed

** Values with same super script are not significantly different

The secondary metabolites produced by the beneficial microbes is having antimicrobial property. Volatile compounds produced by antagonistic fungi and bacteria have been shown to have potential antifungal activities (Alstrom, 2001; Wheatley, 2002; Fernando *et al*., 2005; Kai *et al*., 2006; Zou *et al*., 2007). Antifungal volatiles produced by some fungal strains were used for biological control of plant diseases (Mercier and Manker, 2005; Koitabashi, 2005).Culture filtrate of all the five phyllosphere antagonists were recorded highest inhibition of mycelial growth of *C. siamense* under *in vitro* evaluation. In this study, CKDSF1 (*T. longibrachiatum*) and KVKSF2 (*T. asperellem*) showed 100 per cent mycelial growth inhibition.Culture filtrate of *T. asperellum* which is effective against *Thielaviopsis paradoxa* (Wijesinghe *et al*., 2011). Culture filtrate obtained from *T. longibrachiatum* was effective against *P. infestans* causing tomato late blight disease (Ngo *et al*., 2021) and *Pythium aphanidermatum* (Petrovic *et al*., 2017).

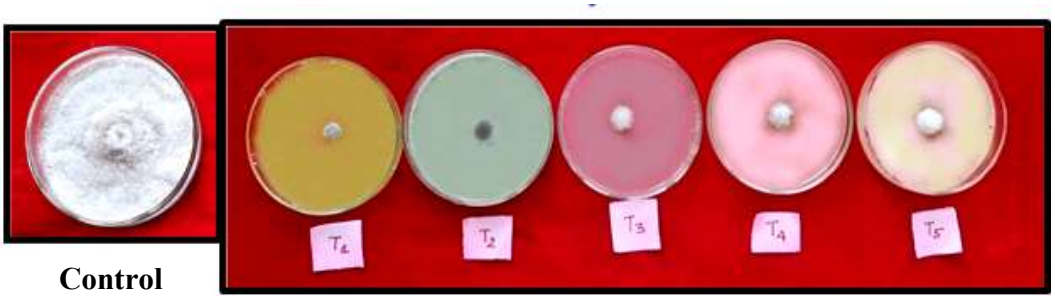
The phyllosphere bacterial isolates, KPCSB1 (*B. amyloliquefaciens*) and MUCSB1 (*B. velezensis*) showed 98.8 and 98 per cent of inhibition of *C. siamense* respectively. Inhibition of *C. dematium* by cell free culture filtrate of *B. amyloliquefaciens* was reported by Yoshida *et al.,* (2000). Cultutre filtrate of endophytic strain of *B. amyloliquefaciens* was effective in controlling soft rot disease in Kiwi fruit caused by *Botryosphaeria dothidea* (Pang *et al*., 2021).

Cell free culture filtrates of *B. velezensis*is effective in plant disease management (Chen *et al*., 2020; Prasanna *et al*., 2021).Compared to other four antagonists NKCSY1 (*Candida tropicalis*) showed lesser inhibition percentage (86.6 per cent).The efficacy of cell free culture filtrates of *C. tropicalis* against plant pathogens is nowhere reported. But in this study, it is proved that the secondary metabolites produced by *C. tropicalis* is having antifungal property and showed mycelial inhibition of *Colletotrichum siamense*.

4.10 EFFECT OF PHYLLOSPHERE ANTAGONISTS ON EARLY GROWTH PROMOTION OF COWPEA

The results of seed treatment were given in the table (4.21). All the five promising phyllosphere isolates and other treatments resulted in 100 per cent germination of cowpea seeds and 66.6 per cent of germination in the control. The speed of germination also varied

Plate 4.13 Effect of secondary metabolites of the phyllopshere antagonists on *C. siamense*



Control **Inhibition of pathogen by secondary metabolites**

Plate 4.14 Effect of phyllosphere antagonists on seed germination and vigour of cowpea

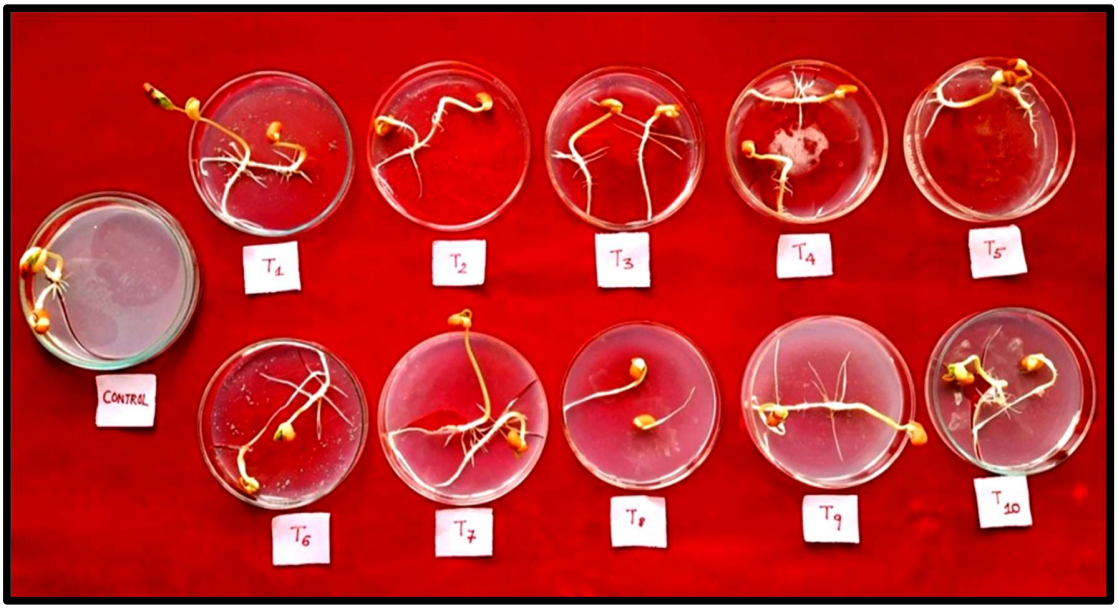
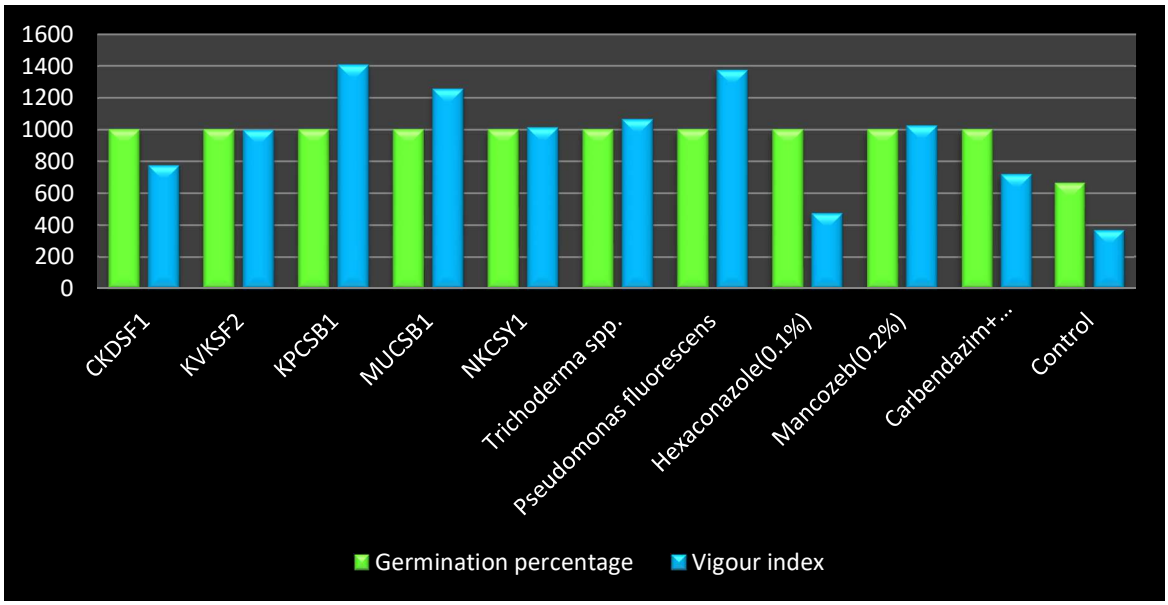


Fig 4.15 Effect of phyllosphere antagonists on early growth promotion of cowpea



differently in treatments. All the treated seeds showed faster germination (one day after sowing) except in the control (3 days for germination) (Plate 4.18). High seedling vigour index was observed in *B. amyloliquefaciens* (T_3) (1406^a), followed by *Pseudomonas fluorescens* (T_7) (1372^b), *B. velezensis* (T_4) (1254^c), *Trichoderma* sp. (T_6) (1064^d), T_9 (1024^e), *Candida tropicalis* (T_5) (1013^e), *T. longibrachiatum* (T_1) (997^e), *T. asperellem* (T_2) (775^f), Carbendazim+ Mancozeb (T_{10}) (720^g), Mancozeb (T_9) (474^h) and control (T_{11}) (368^i) respectively (Fig. 4.15).

Table 4.21 Effect of phyllosphere antagonists on seed germination and vigour index

Treatment	Germination percentage	Days taken for germination	Vigour index
T_1- *T. longibrachiatum*	100	1.0^a	775^f
T_2- *T. asperellem*	100	1.33^{ab}	997^e
T_3- *B. amyloliquefaciens*	100	1.0^a	1406^a
T_4- *B. velezensis*	100	1.66^{ab}	1254^c
T_5- *Candida tropicalis*	100	1.33^{ab}	1013^e
T_6- *Trichoderma* sp.(2% KAU)	100	1.0^a	1064^d
T_7- *Pseudomonas fluorescens* (2% KAU)	100	1.33^{ab}	1372^b
T_8- Hexaconazole (0.1%)	100	1.66^{ab}	474^h
T_9- Mancozeb (0.25%)	100	1.0^a	1024^e
T_{10}- Carbendazim+ Mancozeb (0.2%)	100	1.33^{ab}	720^g
T_{11}- Control	66.6	2.66^b	368^i

* Values with same super script are not significantly different

The plant- microbe relationship and their mutual benefits are very well known. Moreover, most of the studies on phyllosphere microbes have proved their prominent role in plant growth promotion (Zhang *et al*., 2015; Shang *et al*., 2020; Annadurai *et al*., 2020). Further, it will be more beneficial if the phyllosphere antagonist may be used as biocontrol possess growth promoting ability also.

Apart from disease suppression phyllosphere antagonists plays an import role in growth promotion in plants. *T. longibrachiatum* showed an increased seedling vigour in

bioprimed cowpea seeds by increased shoot and root length compared to untreated seeds (control).This may be due to increased root surface contact with soil, which improve nutrient acquisition capacity (Ma *et al*., 2001; Brown *et al*., 2013) and production auxins which induce the activity of plasma membrane H+ ATPase (Haruta *et al*., 2015). *T. asperellem* bioprimed seeds caused enhanced plant growth in various crops (Singh *et al*., 2016; Lopez - Coria *et al*., 2016; Shang *et al*., 2020).

Enhanced seedling vigour index by increasing shoot and root growth was achieved by *B. amyloliquefaciens* (Gowtham *et al.,* 2018).The seeds treated with *B. velezensis* was recorded second highest in seedling vigour index with good germination percentage. *B. velezensis* is an effective bioagent aiding growth promotion in plants along with biocontrol efficacy (Jiang *et al*., 2018; Fan *et al*., 2018; Myo *et al*., 2018).

This positive effect on plant growth may be due to secretion of substances like indole-3-acetic acid and ammonia).*B. velezensis* could increase the exudation of organic carbon and promote root growth also benefit plant growth by nutrient uptake and secreting secondary metabolites such as indole-3-acetic acid to promote the system development of plant roots (Talboys *et al*., 2014; Meng *et al*., 2016; Kim *et al*., 2017).

The phyllosphere antagonistic yeast *C. tropicalis* showed higher vigour index in seedlings. There are many reports on *C. tropicalis,* which act as biofertilizer and enhanceplant growth by enhancing availability of nutrients by solubilization (El- Tarabily and Sivasithamparan, 2006; Botha *et al*., 2011; Amprayn *et al*., 2012; Annadurai *et al*., 2020).

4.11 FIELD EVALUATION OF SELECTED PROMISING PHYLLOSPHERE ANTAGONISTS AGAINST COWPEA ANTHRACNOSE

The efficacy of promising phyllosphere antagonists against cowpea anthracnose was evaluated in comparison with reference cultures and commonly used fungicides (3.7).

4.11.1 Effect of phyllosphere antagonists on plant height and number of main branches of cowpea

Observations on height of plants and number of main branches were recorded at 15, 30 and 45 days after spraying (Table. 4.22).There was no significant difference among the

Fig 4.16 Effect of phyllsophere antagonists on plant height and number of main branches of cowpea

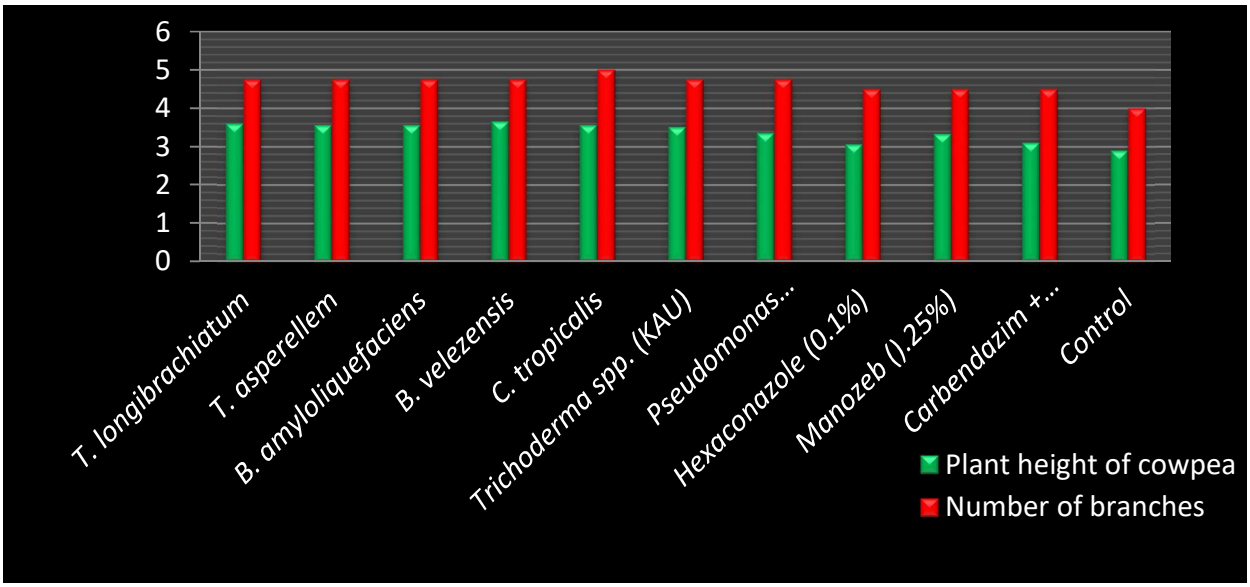
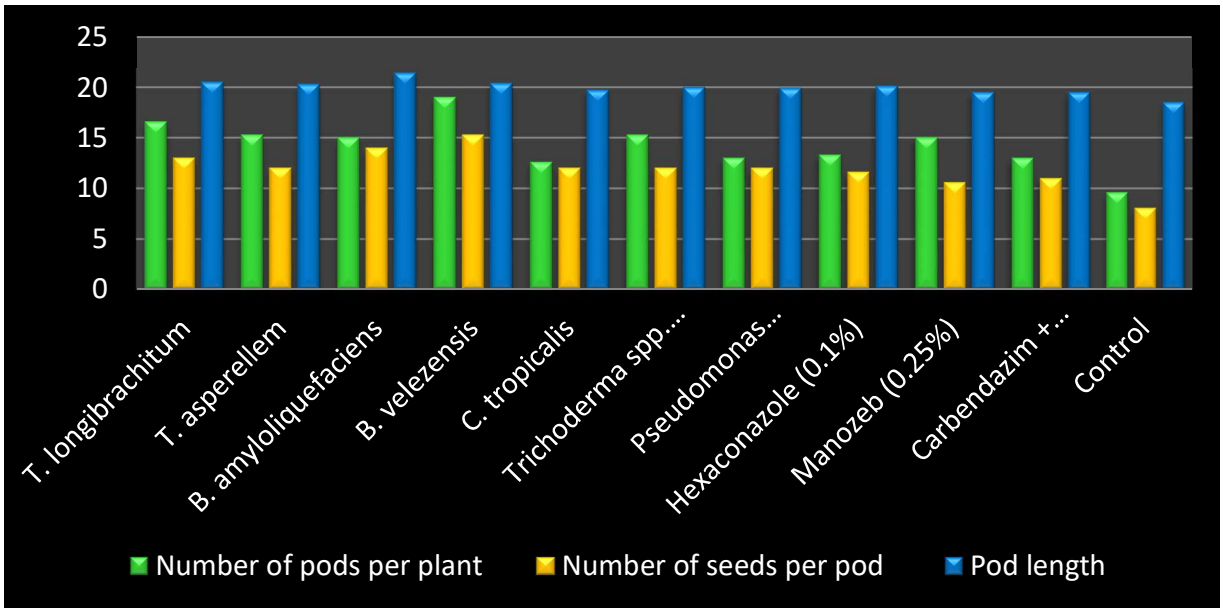


Fig 4.17 Effect phyllosphere antagonists on number of pods per plant, number of seeds per pod and pod length (cm)



treatments at each stage of observation. Plants in the control were shorter among treatments. Plants in *B. velezensis* T_4 (3.66) were the tallest one followed by *T. longibrachiatum* (T_1) (3.6), *T. asperellem* (T_2), *B. amyloliquefaciens* (T_3), *Candida tropicalis* (T_5) (3.56) (Fig 4.16).

Cowpea variety 'Anashwara' is a semi trailing type, but under shade conditions plants grow taller compared to open field (Ajay Gokul and Abdul Hakkim, 2015) obviously, since this study was conducted under rainshelter all the plants grew taller. This

may be the reason forlack of significant difference among the treatments with regard to plant height, which is contradictory to earlier reports (Sasirekha *et al*., 2012). But the data in Table 4.21 shows that, beneficial microbes including phyllosphere antagonists and other biocontrol agents used in this study exert growth promotion in cowpea though the effect is masked to a great extent by the shade.

In this study plants were challenge inoculated with the pathogen *C. siamense,* which hindered the growth to a considerable extent. Along with the tendency to grow taller under shade, the plants had to spend much energy towards the defense responses against the pathogen also. Hence, the growth promoting effect of these beneficial microbes has probably been masked to some extent (Glick, 2012) as observed in plants which are affected with anthracnose disease. Phyllosphere antagonists and other biocontrol agents caused development of systemic resistance upon subsequent sprays, so slightly higher number of main branches was recorded in those treatments.

4.8.1 Effect of phyllosphere antagonists on number of pods per plant, seeds per podand pod length (cm) of cowpea

The observations were recorded in each harvest (Table. 4.23). There was significant difference among the treatments. Highest number of pods per plant was recorded in *B. velezensis* (T_4) (19^a) and less number of pods harvested from plants in control (9.6^c). The statistical analysis of data showed that all treatments were found significantly effective and significant over control (Table 4.23).There was no significant difference among the treatments in case of number of seeds per pod and pod length (cm). However, the statistical analysis of data showed that all treatments were found significantly effective and significant over control (Fig 4.17).

4.8.2 Effect of phyllosphere antagonists on fresh weight of pod (in grams), earlyflowering and duration of harvesting

Observations on fresh weight of pod (in grams) were recorded in each harvest (Table. 4.24). There was no significant difference among the treatments. Observations on effect of phyllosphere antagonists on early flowering and last harvest was recorded (Table. 4.20). The values are analyzed in ascending orders. There was no significant difference among the treatments.

Table 4.22 Effect of phyllosphere antagonists on plant height and number of main branches of cowpea under rainshelter

Treatment	Plant height (in meters)				Number of main branches			
	15 DAS	30 DAS	45 DAS	Per cent +/- over control (45 DAS)	15 DAS	30 DAS	45 DAS	Per cent +/- over control (45 DAS)
T.longibrachiatum (T_1)	2.86	3.2	3.6	+0.7	3	3.75	4.75	+0.75
T. asperellem (T_2)	2.76	3	3.56	+0.66	3	4	4.75	+0.75
B.amyloliquefaciens(T_3)	2.86	3	3.56	+0.66	3	4	4.75	+0.75
B. velezensis (T_4)	2.83	3	3.66	+0.76	3	4	4.75	+0.75
C. tropicalis (T_5)	2.86	3	3.56	+0.66	3	4.25	5	+1.0
Trichoderma sp.(2% KAU) (T_6)	2.6	3.3	3.51	+0.61	3	3.75	4.75	+0.75
P.fluorescens(2%KAU) (T_7)	2.6	3.1	3.36	+0.46	3	4	4.75	+0.75
Hexaconazole(0.1%) (T_8)	2.6	3	3.06	+0.16	3	3.75	4.5	+0.5
Mancozeb (0.25%) (T_9)	2.56	3.1	3.33	+0.43	3	3.75	4.5	+0.5
Carbendazim+ Mancozeb (0.2%) (T_{10})	2.56	2.8	3.1	+0.2	3	3.75	4.5	+0.5
Control (T_{11})	2.36	2.7	2.9	-	3	3.75	4	--
CD(0.05)	NS							

DAS – Days after spraying, NS – Non significant

Fig. 4.18 Effect phyllosphere antagonists on fresh weight of pod (grams)

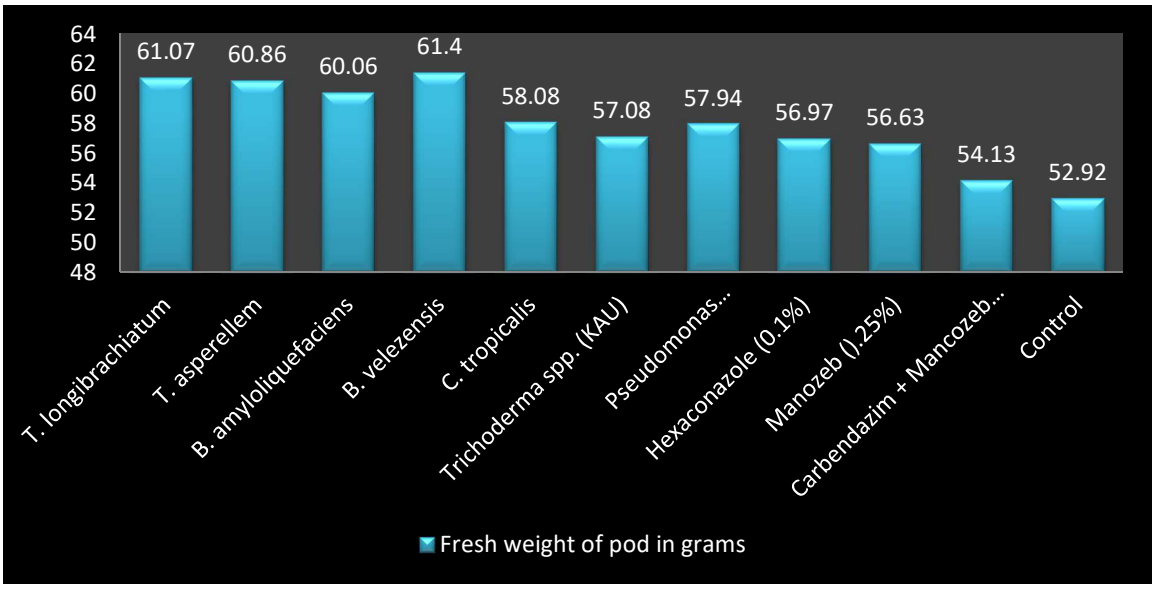


Fig. 4.19 Effect phyllosphere antagonists on duration of harvesting (days)

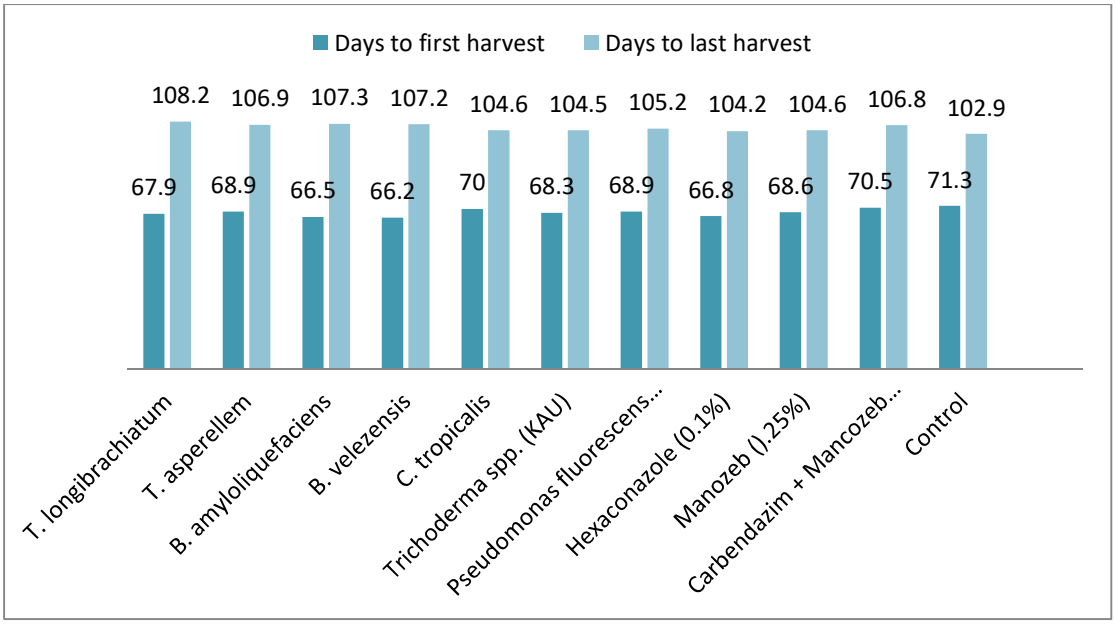


Table 4.23 Effect of phyllosphere antagonists on number of pods per plant, numberof seeds per pod and pod length (cm) of cowpea

Treatment	Number of pods per plant	Per cent +/- over control	Number of seeds per pod	Per cent +/- over control	Pod length (cm)	Per cent +/- over control
T.longibrachiatum (T_1)	*16.6[b]	+7.0	13.0	+4.34	20.5	+2.0
T. asperellem (T_2)	15. [b]	+5.7	12.0	+3.34	20.3	+1.8
B.amyloliquefaciens(T_3)	15[b]	+5.4	14.0	+5.34	21.4	+2.9
B. velezensis (T_4)	19[a]	+9.4	15.3	+6.64	20.4	+1.9
C. tropicalis (T_5)	12.6[bc]	+3.0	12.0	+3.34	19.7	+1.2
Trichoderma sp. (2% KAU) (T_6)	15.3[b]	+5.7	12.0	+3.34	20.0	+1.6
P.fluorescens(2%KAU) (T_7)	13[bc]	+3.4	12.0	+3.34	19.9	+1.4
Hexaconazole(0.1%) (T_8)	13.3[bc]	+3.7	11.6	+2.94	20.1	+1.6
Mancozeb (0.25%) (T_9)	15[b]	+5.4	10.6	+1.94	19.5	+1.0
Carbendazim+ Mancozeb (0.2%) (T_{10})	13[bc]	+3.4	11.0	+2.34	19.5	+1.0
Control (T_{11})	9.6[c]	--	8.66	--	18.5	--
CD(0.05)	S		NS			

S- Significant , NS - Non significant

* Values with same super script are not significantly different

Table 4.24 Effect of phyllosphere antagonists on fresh weight and total pod yield(grams) of cowpea

Treatment	Fresh pod weight (g)	Per cent +/- over control	Total yield (g)	Days to first harvest	Per cent +/- over control	Days to last harvest	Per cent +/- over control
T.longibrachiatum (T_1)	61.07	+8.15	*422.3^{ab}	67.9	- 3.4	108.2	- 5.3
T. asperellem (T_2)	60.86	+7.94	427.7a	68.9	- 2.4	106.9	- 4.0
B.amyloliquefaciens(T_3)	60.06	+7.14	419.5ab	66.5	- 4.8	107.3	- 4.4
B. velezensis (T_4)	61.4	+8.48	415.0abc	66.2	- 5.1	107.2	- 4.3
C. tropicalis (T_5)	58.08	+5.16	419.5ab	70.0	- 1.3	104.6	- 1.7
Trichoderma sp. (2% KAU) (T_6)	57.08	+4.12	406.3abc	68.3	- 3.0	104.5	- 1.6
P.fluorescens(2% KAU) (T_7)	57.94	+5.02	398.6bcd	68.9	- 2.4	105.2	- 2.3
Hexaconazole (0.1%) (T_8)	56. 97	+4.05	403.7abc	66.8	- 4.5	104.2	-1.3
Mancozeb (0.25%) (T_9)	56.63	+3.71	400.0bcd	68.6	- 2.7	104.6	- 1.7
Carbendazim+ Mancozeb (0.2%) (T_{10})	54.13	+1.2	392.4cd	70.5	- 0.8	106.8	- 3.9
Control (T_{11})	52.92	--	377.4d	71.3	--	102.9	--
CD(0.05)		NS		S	NS		NS

NS - Non significant, S= significant

* Values with same super script are not significantly different

4.8.3 Effect of phyllosphere antagonists on yield of cowpea

Observations on total yield (in grams) of cowpea pods were recorded in each harvest and yield from each harvest is recorded (Table. 4.23). There was significant difference among the treatments. Highest pod yield was recorded in all the treatments except in plants in the control. *T. asperellem* (T_2) (427.7^{a}), followed by *T. longibrachiatum* (T_1) (422.3^{ab}), *B.amyloliquefaciens* (T_3) and *Trichoderma* sp. (KAU)(T_6) are on par with each other (419.5^{ab}) followed by *B. velezensis* (T_4)(415.0^{abc}), Hexaconazole (0.1%) (T_8),

Mancozeb (0.25%) (T_9)(403.7^{abc}), Carbendazim+ Mancozeb (0.2%) (T_{10}) (392.4^{cd}), low yield wasobserved in plants in the control (T_{11})(377.4^{d}).

In terms of pod yield more than 60 per cent increase in number pods per plant was observed in plants treated with *B. velezensis*, followed by more than 35 per cent increase was recorded by *T. longibrachiatum*, *T. asperellem*, *B. amyloliquefaciens* and 20 per cent increase in *C. tropicalis*. Biometric observations recorded in the present study strongly indicated that the role of phyllosphere microbes as plant growth promotion expressed in cowpea. Fifty per cent increase in pod yield was observed in isolate *T. asperellem* and more than 40 per cent increase was recorded in *T.longibrachiatum*, *B. amyloliquefaciens* and *C. tropicalis* and more than 35 per cent increase was recorded in isolate *B. velezensis*. Even though the effect of antagonistic phyllosphere microbes were not well expressed on plant growth, there is significant increase in terms of productivity. The collective effect of these microbes on growth promotion and disease reduction has brought about this result. Phyllosphere microorganisms as well as biocontrol agents showed increased productivity by improving plant growth, it is mainly because of reduced disease severity due to production defence related enzymes. Hence, the results shows that, plant productivity can be increased by application of beneficial microbes (Rocha *et al*., 2019; Lopes *et al*., 2021).

Many studies revealed that phyllosphere microflora plays an important role in plant growth promotion and yield in various agricultural crops by means of production of different compounds against biotic and abiotic stress, which includes ACC deaminase enzymes, auxins, secondary metabloites *etc.* (Chaudary *et al*., 2007; Abadi *et al*., 2020; Sharath *et al*., 2021).

4.8.4 Effect of phyllosphere microbes against cowpea anthracnose disease caused by *C. siamense*

The anthracnose disease incidence and severity of disease upon challenge inoculation was recorded at periodic intervals (Table. 4.25). The disease incidence was varied from 25 to 100 per cent from at 15, 30, 45 DAS and there was significant difference among the treatments over the control. The disease severity was varied from

0.53 to 71.0 per cent from at 15, 30, 45 DAS and there was no significant difference among the treatments. At 15DAS lowest disease incidence was observed in

T.longibrachiatum (T_1), *C. tropicalis* (T_5) and Mancozeb (0.25%) (T_9). hese treatments were on par (8.3^a) and higher disease incidence was recorded in plants in the control (T_{11}) (47.2^c). At 30 DAS, lower disease incidence was recorded in *Trichoderma* sp. (2% KAU) (T_6) , *P.fluorescens* (2% KAU) (T_7), Hexaconazole (0.1%) (T_8) and Mancozeb (0.25%) (T_9) and they were on par. At 15 DAS and 30 DAS lowest disease severity was observed in all the treatments except in plants in control. In control, higher disease severity was recorded in control plants (23.83^b) at 15 DAS and (44.40^b) at 30 DAS.

At 45 DAS, lower disease incidence was recorded in *C. tropicalis* (T_5) (58.3^a) and Carbendazim+ Mancozeb (0.2%) (T_{10}) (55.5^a) and they were on par and higher disease incidence was recorded in T_{11} (control) (100^d). However, there were significant reduction in disease severity in all the treatments at 45 DAS, except *T.longibrachiatum* (T_1) (26.6^b) and T_{11} (control) (71.0^c) (Fig 4.19).

Table 4.25 Effect of phyllsophere microbes on anthracnose disease of cowpea

Treatments details	PDI of anthracnose			PDS of anthracnose		
	15 DAS	30 DAS	45 DAS	15 DAS	30 DAS	45 DAS
T.longibrachiatum (T_1)	*8.33^{a}	66.6^{c}	86.0^{C}	1.1^{a}	6.63^{a}	26.6^{b}
T. asperellem (T_2)	16.6^{b}	47.2^{b}	74.9^{bc}	2.2^{a}	7.2^{a}	12.2^{ab}
B.amyloliquefaciens(T_3)	25.0^{bc}	55.5^{bc}	86^{C}	5.0^{a}	6.1^{a}	13.8^{ab}
B. velezensis (T_4)	16.6^{b}	52.7^{bc}	83.3^{bc}	2.2^{a}	4.4^{a}	13.8^{ab}
C. tropicalis (T_5)	8.33^{a}	41.6^{b}	58.3^{A}	0.53^{a}	4.4^{a}	6.63^{ab}
Trichoderma sp.(2% KAU) (T_6)	16.6^{b}	33.3^{a}	80.0^{bc}	2.2^{A}	3.3^{a}	11.1^{ab}
P.fluorescens(2%KAU) (T_7)	16.6^{b}	33.3^{a}	74.9^{bc}	2.2^{A}	7.76^{a}	9.96^{ab}
Hexaconazole(0.1%) (T_8)	25.0^{bc}	36.1^{ab}	69.4^{B}	5.0^{A}	8.33^{a}	8.86^{ab}
Mancozeb (0.25%) (T_9)	8.33^{a}	38.8^{ab}	80.0^{bc}	1.6^{A}	6.1^{a}	7.76^{ab}
Carbendazim+Mancozeb (0.2%) (T_{10})	16.6^{b}	47.2^{b}	55.5^{A}	2.2^{A}	4.96^{a}	6.06^{a}
Control (T_{11})	47.2^{c}	86.0^{d}	100^{D}	23.83^{B}	44.40^{b}	71.0^{c}
CD(0.05)	S (12.6)	S (13.2)	S (11.5)	S (8.42)	S (13.1)	S(5.69)

S- Significant

* Values in the parenthesis with same super script are not significantly different

Fig. 4.20 Effect phyllosphere microbes on pod yield

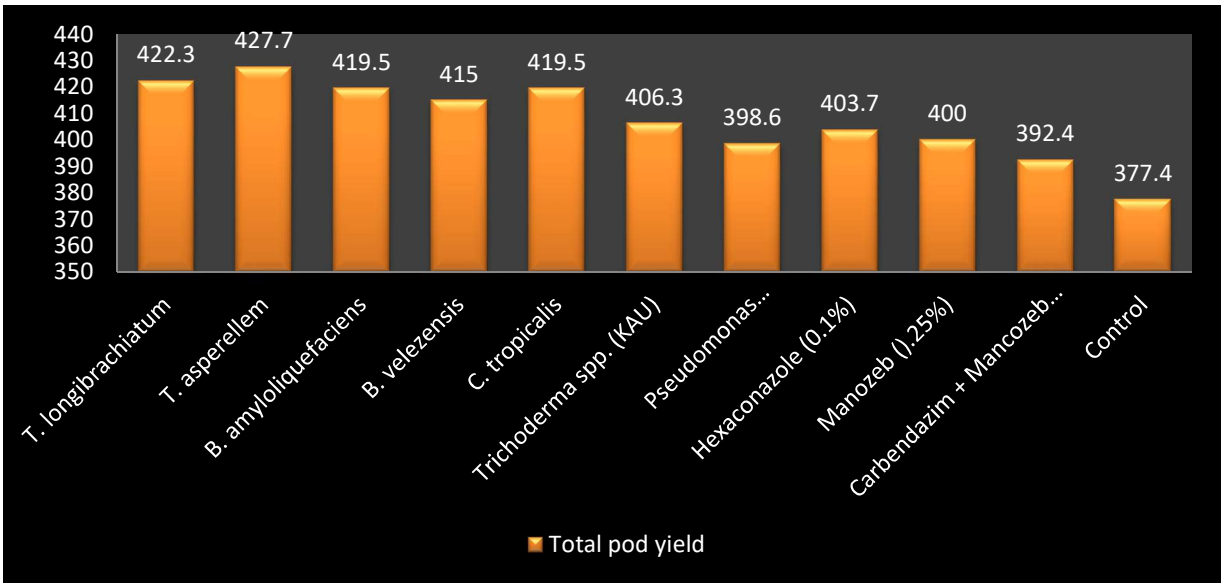


Fig.4.21 Effect phyllosphere microbes on anthracnose disease incidence

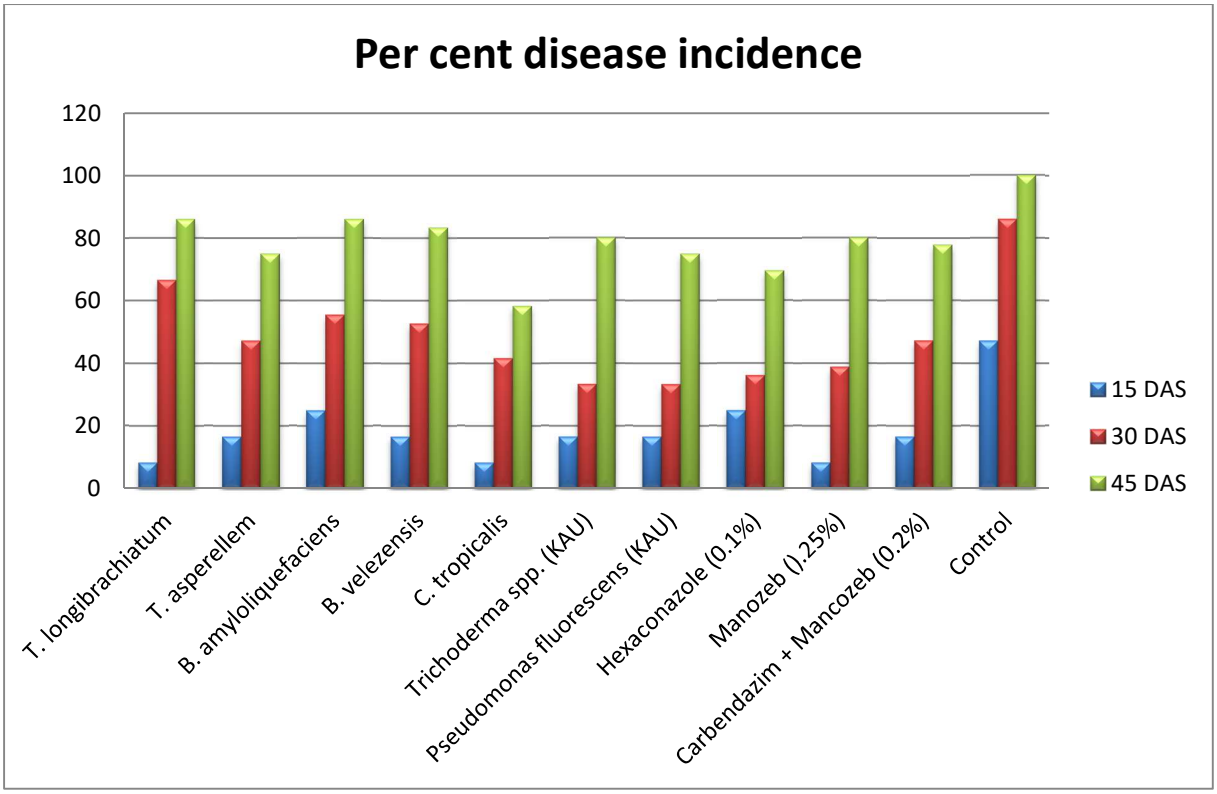
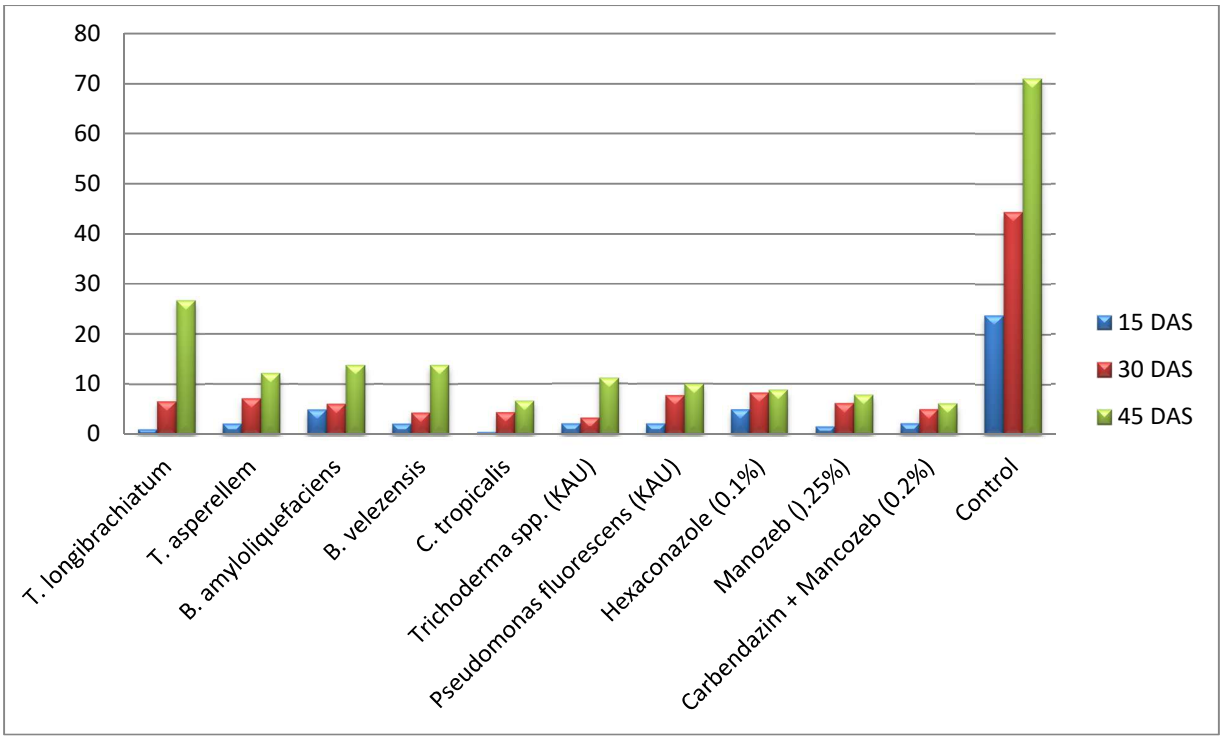


Fig.4.22 Effect phyllosphere microbes on anthracnose disease severity



Field evaluation of phyllosphere antagonists against cowpea anthracnose disease revealed that phyllosphere antagonists reflected positive effects on treated plants such as anthracnose disease tolerance and maintenance of plant growth and productivity. Among microbial treatments phyllosphere antagonists were more effective compared to conventional biocontrol agents such as *Trichoderma* sp. and *Pseudomonas fluorescens*. Since the phyllosphere antagonists are associated with cowpea itself, they can colonize and establish on cowpea plant surface more easily compared to other biocontrol agents (O'brien and Lindow, 1989).

Out of eleven treatments, plants treated with phyllosphere antagonists were recorded good plant growth and yield. But the plants treated with fungicides as well as, T_5 (*C. tropicalis*) were recovered from anthracnose more quickly than other treatments. Among fungicides, the best treatment was T_{10} (Carbendazim + Mancozeb) (combination - contact + systemic) and other two, T_8 (Hexaconazole) (systemic fungicide) and(Mancozeb) (contact fungicide) are also found effective. Among the five phyllsophere antagonists, bacterial isolate T_3 (*B. amyloliquefaciens*) and fungal isolate T_2 (*T. asperellem*) was found best among the treatments in terms of their plant growth promotion, yield parameters as well as disease suppressing ability.

Among phyllosphere antagonists, plants treated with phyllosphere yeast *C. tropicalis* (T_5) was found to be an effective biocontrol agent against anthracnose caused by *Colletotrichum siamense.* It is contrary to *in vitro* evaluation since it showed lowest per cent inhibition of pathogen compared to other antagonists. Along with this, it improved plant growth and yield with superior quality pods. The antagonistic ability of

C. tropicalis may be due to the production of anti pathogen diffusible metabolites and cell wall degrading enzymes (El Mehalawy, 2004; El Tarabily and Sivasithamparan, 2005). It act as a biofertilizer and indirectly boost plant growth, in addition to it's antagonistic qualities and found to promote cowpea growth and productivity (El Tarabily and Sivasithamparan, 2006; Botha, 2011; Nour and Tolba, 2015; Annadurai, 2020).

T. asperellem performed best under *in vitro* and *in vivo* evaluations. It is an effective mycoparasite of *C. gloeosporioides* (Quiroz *et al*., 2015; Shang *et al*., 2020). In the present study, fungus *T. asperellem* showed good yield and growth in cowpea plants. The fungus

also improves the plant's health by increasing root surface contact with the soil, which aids in nutrient absorption capacity (Ma *et al*., 2001; Brown *et al*., 2013). Thesynthesis of auxins (IAA), which promote the activity of plasma membrane H+ ATPase, improves plant development in *T. asperellem* treated plants (Haruta *et al*., 2015; Lopez- Coria *et al*., 2016). In addition, it boosts vegetable growth and yield (Singh *et al*., 2016).

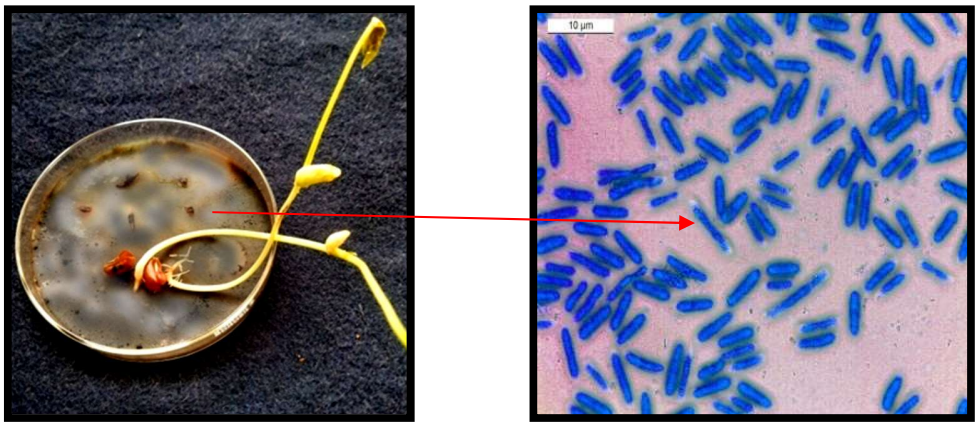
B. amyloliquefaciens and *B. velezensis* showed low disease severity in cowpea plants. Both species grouped under *B. subtilis* species complex (Fan *et al*., 2017; Rabbee *et al*., 2019). *B. amyloliquefaciens* isolated from phyllosphere showed antagonism towards various pathogens *viz. Cercospora beticola* (Esh *et al.,* 2011), *C. gloeosporioides* (Mochizuki *et al*., 2012), *Blumeria graminis* f. sp. *tritici* (Hongfeng *et al*., 2014) and *Colletotrichum truncatum* (Gowtham *et al*., 2018) and there are recent reports on probiotic nature (Li *et al*., 2018) of this organism and thus safely recommend it to the farmers. *B. velezensis* is an effective emerging biocontrol agent for various plant diseases. It is a potential biocontrol agent which produces various anti pathogenic enzymes such as cellulases, proteases and peroxidases (Chen *et al*., 2020). It is used as biocontrol agent against rice blast disease (Chen *et al*., 2020), mango athracnose (Jin *et al*., 2020), walnut anthracnose (Choub *et al*., 2021) and cell free culture filtrates used against blast and bacterial blight disease of rice (Prasanna *et al*., 2021). Both these bacterial isolates, recorded superior plant growth and yield with good quality pods and seeds. *T. longibrachiatum* (T_1) also resulted reasonable performance in reducing disease severity under field conditions and enhanced plant growth with good yield. *T. longibrachiatum* can be considered to be a promising bio-control agent (Zhang *et al*., 2015) and growth promotion in various crops (Zhang *et al*., 2016; Montesinos *et al*., 2019). Based on performance under *in vitro* and *in vivo* studies, as well as safe and non pathogenic nature, we could recommend that, *B. amyloliquefaciens* and *T. asperellem* are promising phyllosphere microorganisms against cowpea anthracnose disease.

4.8 STUDY ON SEED BORNE NATURE OF PATHOGEN CAUSING COWPEAANTHRACNOSE DISEASE

The infected seeds collected from field were germinated on PDA. Mycelial growth of *Colletotrichum* sp. from seeds was observed in PDA plate. The cultural and morphological characterization was studied and it was that the isolate is *C. siamense.*

This study revealed that the pathogen *C. saimense* causing anthracnose disease in cowpea is seed borne in nature (Plate 4.15). Hence, this might affect cowpea growers to manage the disease and seeds from healthy plants should be recommended for cultivation. There are so many evidences of seed borne infection of pathogen causing cowpea anthracnose disease (Suryanarayana, 1978; Emechebe and McDonald, 1979).

Plate 4.15 Seed borne nature of *C. siamense*



Conidia of *C. siamense*

Summary

5. SUMMARY

Cowpea anthracnose is one of the major fungal diseases faced by cowpea farmers of Kerala, anthracnose disease caused by *Colletotrichum* fungus is the most serious one, which cause reduced yield. Since it affects reproductive stage of the crop compared to vegetative stage. Under severe conditions it also spreads to pods and affects market quality as well as seeds get deformed. For the fast recovery from disease, chemical fungicides are commonly used by the farmers, since it affects reproductive stage of the crop compared to vegetative stage. Under severe conditions it also spreads to pods and affects market quality as well as seeds get deformed. Moreover, continuous use of chemical fungicides results in deleterious effects in the ecosystem and also reduce the population of microflora in the phyllosphere. Here comes the significance of biocontrol agents, plays an important role in sustainable agriculture and eco-friendly in nature. Disease which affects aerial plant parts in which the effectiveness of conventional biocontrol agents will be less and their extent of successful colonization is limited. Hence, studies on phyllosphere antagonists become more important, as they are having beneficial effects on plants by inducing systemic resistance against plant diseases and growth promoting ability in plants. Hence, the present investigation was carried out with the objective of harnessing the native of phyllosphere microbes of cowpea for the management of anthracnose disease of cowpea. The salient findings of the study are summarized below:

1. The pathogen causing anthracnose of cowpea was isolated from various places and its pathogenicity established. The cultural and morphological characters of the pathogen isolates were studied. The isolates were confirmed as *Colletotrichum siamense,* three isolates of *Colletotrichum* sp. and other pathogens such as *Ectophoma multirostrata, Curvularia verruculosa* are found infecting cowpea and causing anthracnose disease symptoms. From these isolates *C. siamense* is used for further evaluation.
2. Enumeration of phyllosphere microorganisms from different parts of cowpea plants grown at various locations of the major cowpea growing areas of the state revealed the predominance of bacteria and fungi compared to fluorescent pseudomonads and yeasts.

3. The phyllosphere antagonists population is more in stems and leaves compared to other parts of the plant. Since the anthracnose infection and pathogen load is more on stem and leaves compared to other plant parts, which proves the theory of co-evolution between pathogens and it's antagonists. Altogether, 183 phyllosphere isolates were collected including 85 bacteria, 84 fungi, 7 fluorescent pseudomonads and 7 yeasts.
4. Out of 183 phyllosphere isolates subjected to preliminary screening, 75 selected for secondary screening and from this, 41 were found to be antagonistic to the pathogen. Among the phyllosphere antagonists, there were more fungi (25), compared to bacteria (15) and yeasts (1).
5. Out of 41 antagonists, 25 exerted more than 60 per cent *in vitro* inhibition of the pathogen, and these included 15 fungi, 9 bacteria and one yeast isolate. Among this superior five isolates including two fungus, two bacteria and one yeast were selected for further evaluation to study their efficacy under field conditions.
6. The effect of phyllsophere antagonists on seed germination and seedling vigour was studied by treating the seeds using agar plate method. All the five antagonists showed good seedling vigour, among this the two bacterial isolates, *B. amyloliquefaciens* and *B. velezensis* recorded maximum vigour index compared to reference cultures *viz. Trichoderma* sp. and *Pseudomonas fluorescens.*
7. The effect of secondary metabolites of isolates were studied by using cell free culture filtrate method and the fungal isolates *T. longibrachiatum* and *T. asperellem* recorded complete inhibition of mycelial growth of *C. siamense*, it was followed by bacterial and yeast isolates.
8. Defence related enzymes activity in plants were analyzed to evaluate induced systemic resistance in plants treated with phyllosphere antagonists. Profound increase in peroxidase and phenylalanine ammonia lyase activity was observed in plants treated with *B. amyloliquefaciens*. The plants treated with *C. tropicalis* recorded higher activity polyphenol oxidase. All the five antagonists showed increasing trend in PO, PPO, PAL activity from 0 to 7 days of inoculation.
9. Field evaluation of phyllosphere antagonists yielded positive results. Field experiment revealed efficiency of promising phyllosphere antagonists in the

management of cowpea anthracnose. Efficacy of treatments varied among the three phases of the field trial. In the first phase *viz.* due to seed treatment and first spraying, delay in incidence of disease was observed in all the treatments except in the control.

10. Based on *in vitro* and *in vivo* studies, it is found that phyllosphere angtagonists are very effective in plant growth and disease management and the best performance was showed by *B. amyloliquefaciens* and *T. asperellem* and they can safely recommend to the farmers as formulated product for the management of cowpea anthracnose disease.

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Appendices

APPENDIX – 1

MEDIA COMPOSITION

Potato Dextrose Agar (PDA)

Potato	-	200g
Agar	-	20g
Dextrose	-	20 g
Distilled water	-	1000ml
pH	-	7.0

Nutrient Agar (NA)

Peptone	-	5g
Beef extract	-	1g
Sodium chloride	-	5g
Agar	-	15g
Distilled water	-	1000ml

King's B Medium

Peptone	-	20g
Glycerol	-	10g
K_2HPO_4	-	10g
$MgSO_4 .7H_2O$	-	1.5g
Agar	-	15g
Distilled water	-	1000ml
pH	-	7.2-7.4

Kenknight's Agar medium (KAM)

Glucose	-	1g
KH_2PO_4	-	0.1g
NaNO3	-	0.1g
KCl	-	0.1g
$MgSO_4.7H_2O$	-	0.1g
Agar	-	20g
Distilled water	-	1000ml

Martin Rose Bengal Streptomycin Agar (MRBA)

KH_2PO_4	-	1g
$MgSO_4.7H_2O$	-	0.5g
Peptone	-	5g
Dextrose	-	10g
Rose Bengal	-	0.03g
Streptomycin	-	30g
Agar	-	20g
Distilled water	-	1000ml

Glucose yeast extract peptone agar

Glucose	-	20g
Yeast extract	-	5g
Peptone	-	5g
Agar	-	20g
Distilled water	-	1000ml

APPENDIX –II

ITS SEQUENCES OF VHT (*Colletotrichum siamense*)

GCCGAACCTGCGGAGGGATCATTACTGAGTTTACGCTCTACAACCCTTTGTGAAC
ATACCTATAACTGTTGCTTCGGCGGGTAGGGTCTCCGCGACCCTCCCGGCCTCCC
GCCTCCGGGCGGGTCGGCGCCCGCCGGAGGATAACCAAACTCTGATTTAACGAC
GTTTCTTCTGAGTGGTACAAGCAAATAATCAAAACTTTTAACAACGGATCTCTTG
GTTCTGGCATCAATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAG
AATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTCTGGC
GGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCTCTGCTTGGTGTTGGGGC
CCTACAGCTGATGTAAGCCCTCAAAGGTAGTGGCGGACCCTCTCGGA

ITS SEQUENCES OF BNT (*Colletotrichum* sp.)

AGGCTTCGTCACTGACCTCCACGTCCGCCTACTCCTCAGCGCATCGTTTCTACGCT
GAGGGCGAGGTATGGGTGAGACGCTTGAGCGCCATCCATTTTCAGGGCTAGTAC
ATTCGGCAGGTGAGTTGTTACACAGTCCTTAGCGGATTCCGACTTCCATGGCCAC
CGTCCTGCTGTCAAGATGTACTAACACCTTTTGTGGTGTCTGATGAGCGTCTACTC
TGGCACCTTAACCTCGCGTTCGGTTCATCCCGCATCGCCAGTTCTGCTTACCAAAA
ATGGCCCACTAGTGTTGATACATTCGAATGCCCACGTTCAGCTAAGTAACAAGGG
CTTCTTACATATTTAAAGTTTGAGAATGGATGAAGGCAATATAGCGCCCCCGAGT
CCCTAATCATTCGCTTTACCTCATAAAACTGAGTTCAACACTGCTATCCTGAGGG
AAACTTCGGCGGAAACCAGCTACTAGAAGGTTCGATTAGTCTTTCGCCCCCATGC
GCATATTTGACGATCGATTTGCACGTCAGAACCCGCTGCGAGCCTCCACCAGAGT
TTCCTCTGGCTTCACCCTATACACGCATAGTTCACCTTCTTTCGGGTCCAACCCTA
TATGCTCTTACTCAAATCCATCCGAGAACATCAGGATCGGTCGATGATGCGCCGA
AGCTCTCACCTGCGTTCACTTTCATTTCGCGTAGGGGTTTGACACCCGAACACTCG
CACATAAGGTTGACTCCTTGGTCCGTGTTTCAAGACGGGTCGCTGATGACCATTA
CGCCAGCATCCTTGCGGAGCGCGTACCTCAGCCCGCCGAAGGGTATTGTGCAGCG
GGCTATAACACTCCCCGAAGAGAGCTACGTTCCCGAAGCTTTTGTCCCCGACGGC
GAGCTGATGCTGGCCTGAGCCGGCAAAGTGCCCCAGCCGCGAGAGCTGGGTGAT
TCACCGGGCGCAAGTCTGGTCACAAGCGCTTCCCTTTTAACAATTTCACGTGCTGT
TTAACCCTCTTTTCAAAGTGCTTTTCATCTTTCGATCACTCTACTTGTGCGCTATCG
GTCTCTGGCCGGTATTTAGCTTTAGAAGAAATATACCTCCCATTTAGAGCAGCAT
TCCCAAACTACTCGACTCGTCGAAGGAGCTTTACACAGGCTTGGTGTCCAACCGT
ACGGGGCTCTCACCCTCTATGGCGTCCCGTTCCAGGGAACTCGGAAGGCACCGCG
CCAAAAGCATCCTCTGCAAATTACAACTCGGACCCTGGGGGCCAGATTTCAAATT
TGAGCTGTTGCCGCTTCACTCGCCGTTACTGAGGCAAT

ITS SEQUENCES OF OLR (*Colletotrichum* sp.)

AGGCTTCGTCACTGACCTCCACGTCCGCCTACTCCTCAGCGCATCGTTTCTACGCT
GAGGGCGAGGTATGGGTGAGACGCTTGAGCGCCATCCATTTTCAGGGCTAGTAC
ATTCGGCAGGTGAGTTGTTACACAGTCCTTAGCGGATTCCGACTTCCATGGCCAC
CGTCCTGCTGTCAAGATGTACTAACACCTTTTGTGGTGTCTGATGAGCGTCTACTC
TGGCACCTTAACCTCGCGTTCGGTTCATCCCGCATCGCCAGTTCTGCTTACCAAAA
ATGGCCCACTAGTGTTGATACATTCGAATGCCCACGTTCAGCTAAGTAACAAGGG

CTTCTTACATATTTAAAGTTTGAGAATGGATGAAGGCAATATAGCGCCCCCGAGT
CCCTAATCATTCGCTTTACCTCATAAAACTGAGTTCAACACTGCTATCCTGAGGG
AAACTTCGGCGGAAACCAGCTACTAGAAGGTTCGATTAGTCTTTCGCCCCCATGC
GCATATTTGACGATCGATTTGCACGTCAGAACCCGCTGCGAGCCTCCACCAGAGT
TTCCTCTGGCTTCACCCTATACACGCATAGTTCACCTTCTTTCGGGTCCAACCCTA
TATGCTCTTACTCAAATCCATCCGAGAACATCAGGATCGGTCGATGATGCGCCGA
AGCTCTCACCTGCGTTCACTTTCATTTCGCGTAGGGGTTTGACACCCGAACACTCG
CACATAAGGTTGACTCCTTGGTCCGTGTTTCAAGACGGGTCGCTGATGACCATTA
CGCCAGCATCCCTTGCGGAGCGCGTACCTCAGCCCGCCGAAGGGTATTGTGCAGC
GGGCTATAACACTCCCCGAAGAGAGCTACGTTCCCGAAGCTTTTGTCCCCGACGG
CGAGCTGATGCTGGCCTGAGCCGGCAAAGTGCCCCAGCCGCGAGAGCTGGGTGA
TTCACCGGGCGCAAGTCTGGTCACAAGCGCTTCCCTTTTAACAATTTCACGTGCTG
TTTAACCCTCTTTTCAAAGTGCTTTTCATCTTTCGATCACTCTACTTGTGCGCTATC
GGTCTCTGGCCGGTATTTAGCTTTAGAAGAAATATACCTCCCATTTAGAGCAGCA
TTCCCAAACTACTCGACTCGTCGAAGGAGCTTTACACAGGCTTGGTGTCCAACCG
TACGGGGCTCTCACCCTCTATGGCGTCCCGTTCCAGGGAACTCGGAAGGCACCGC
GCCAAAAGCATCCTCTGCAAATTACAACTCGGACCCTGGGGGCCAGATTTCAAAT
TTGAGCTGTTGCCGCTTCACTCGCCGTTACTGA

ITS SEQUENCES OF UDK (*Colletotrichum* sp.)

ATTTAAACGACGTCTCTTCTGAGTGGCACAAGCAAATAATCAAAACTTTTAACAA
CGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATG
TGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCA
GCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTG
GCGTTGGGCTCTCCGCATGTGACCTGCGGCATATCACTAAGCGGAGGAACCTCCG
TAGGTGAACTGCCGCCTACCTCGCCCGGAACCACCGTCTCGGCGCGCCCCACCCG
CCGGCGGACCACCAAATTCTATTTAAACGACGTCTCTTCTGAGTGGCACAAGCAA
ATAATCAAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCA
GCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTG
AACGCACATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTT
CAACCCTCAAGCACCGCTTGGCGTTGGGCCCCTAAAAAATCAATAGGCCCCCACC
ACAAAAAAGTACCCCCCCGCAACAAACTTTGCCCACCAGCTTACGGGCAATTTAA
ACGACGTCTCTTCTGAGTGGCACAAGCAAATAATCAAAACTTTTAACAACGGATC
TCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATT
GCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGCATTC
TGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCCTCAAGCACCGCTTGGCGTTG
GGCTCTCCGCATGTGACCTGCGGCATATCACTAAGCGGAGGAACCTCCGTAGGTG
AACTGCCGCCTACCTCGCCCGGAACCACCGTCTCGGCGCGCCCCACCCGCCGGCG
GACCACCAAATTCTATTTAAACGACGTCTCTTCTGAGTGGCACAAGCAAATAATC
AAAACTTTTAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAA
TGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCA
CATTGCGCCCGCCAGCATTCTGGCGGGCATGCCTGTTCGAGCGTCATTTCAACCC
TCAAGCACCGCTTGGCGTTGGGCCCCTAAAAAATCAATAGGCCCCCACCACAAA
AAAGTACCCCCCCGCAACAAACTTTGCCCACCAGCTTACGGGCA

ITS SEQUENCES OF MLA (*Ectophoma multirostrata*)

CTTCCCGTAGGTGAACCTGCGGAAGGATCATTACCTAGAGTTGTAGGCTTTGCCT
GCTATCTCTTACCCATGTCTTTTGAGTACCTTCGTTTCCTCGGCGGGTCCGCCCGC
CGATTGGACACATTTAAACCCTTTGTAGTTGCAATCAGCGTCTGAAAAACTTTAA
TAGTTACAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAG
CGAAATGCGATAAGTAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGA
ACGCACATTGCGCCCCTTGGTATTCCATGGGGCATGCCTGTTCGAGCGTCATTTGT
ACCTTCAAGCTTTGCTTGGTGTTGGGTGTTTGTCTCGCCTATGCGCGCAGACTCGC
CTCATAACAATTGGCAGCCGGCGTATTGATTTCGGAGCGCAGTACATCTCGCGCT
TTGCACTCATAACGACGACATCCAAAAAGTACATTTTTTACACTCTTGACCTCGG
ATCAG

ITS SEQUENCES OF ALR (*Curvularia verruculosa*)

AAGGCTTCGTCACGAGCCTCCACGCCTGCCTACTCGCCGGGGCGTAAATTTTGCC
CCGGCGGAGGGGTATAGGTGACACGCTTGAGCGCCATCCATTTTCAGGGCTAGTT
CATTCGGCAGGTGAGTTGTTACACACTCCTTAGCGGATTCCGACTTCCATGGCCA
CCGTCCTGCTGTCTAGATGAACCAACACCTTTTGTGGTGTCTGATGAGCGTGTACT
CCGGCACCGTAACCCCTCGTTCGGTTCATCCCGCATCGCCAGTTCTGCTTACCAAA
AATGGCCCACTAATAACGTTTCATTCAAATGCCCGCGTTCAATTAAGTAACAAAG
GGCTTCTTACATATTTAAAGTTTGAGAATAGGTGAAGGTTGTTTCAACCCCCATG
CCTCTAATCATTCGCTTTACCTCATAAAACTGAATACGTTACTGCTATCCTGAGGG
AAACTTCGGCAGGAACCAGCTACTAGATAGTTCGATTAGTCTTTCGCCCCTATGC
CCAAATTTGACGATCGATTTGCACGTCAGAACCGCTGCGAGCCTCCACCAGAGTT
TCCTCTGGCTTCACCCTATTCAAGCATAGTTCACCATCTTTCGGGTCCCAACAGCC
ATGCTCTTACTCAAATCCTTCCGTAAACTTCAGGATCGGTCGATGGTGCGCCCTTG
CGGGTTCCCACCTCCGTTCACTTTCATTACGCGCTCGGGCTTGACACCCAAACACT
CGCATAGATGTTAGACTCCTTGGTCCGTGTTTCAAGACGGGCCCGCTTACAGCCA
TTAC

ITS SEQUENCES OF *Trichoderma longibrachiatum*

TGTGAACCTGCGGAGGGATCATTACCGAGTTTACAACTCCCAAACCCCAATGTGA
ACGTTACCAATCTGTTGCCTCGGCGGGATTCTCATGACCCGGGCGCGTCCCAACC
CCGGATCCCATGGCGCCCGCCGGAGGACCAACTCCAAACTCTTTTTTTCTCTCCGT
CGCGGCTCCCGTCGCGGCTCTGTTTTATTTTTGCTCTGAGCCTTTCTCGGCGACCC
TAGCGGGCGTCTCGAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTG
GCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCAT
GCCTGTCCGAGCGTCATTTCAACCCTCGAACCCCTCCGGGGGGGTCGGCGTTGGGG
ATCGGCCCCTCACCGGGCCGCCCCCGAAATACAGTGGCGGTCTCGCCGCAGCCTC
TCCTGCGCAGTAGTTTGCACACTCGCACCGGGAGCGCGG

ITS SEQUENCES OF *Trichoderma asperellem*

TGAGACGCTTGAGCGCCATCCATTTTCAGGGCTAGTACATTCGGCAGGTGAGTTG
TTACACAGTCCTTAGCGGATTCCAACTTCCATGGCCACCGTCCTGCTGTCAAGAT
GTACTAACGCCTTTTGTGGTGTCTGATGAGCGTCTACTCTGGCACCTTAACCTCGC
GTTCGGTTCATCCCGCATCGCCAGTTCTGCTTACCAAAAATGGCCCACTAATGTTG

ATACATTCGAATGCCCACGTTCAACTAAATAACAAGGGCTTCTTACATATTTAAA
GTTTGAAAATGGATGAAGGCAATATACCGCCCCCGAGTCCCTAATCATTCGCTTT
ACCTCATAAAACTGAGCTCAACACTGCTATTCTGAGGGAAACTTCGGCGGAAACC
AGCTACTAAAAGGTTCGATTACTCTTTCGCCCCCATGCCCATATTTG

16s rDNA SEQUENCES OF *Bacillus amyloliquefaciencs*
TTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCGAACTGAG
AACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTTCTGCCCA
TTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACGTCATCCC
CACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGAATGCTG
GCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGAC
ACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGGGACGTCC
TATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGCGTTGCTTC
GAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTT
CAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCTGCAGCACT
AAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTGGACTACCA
GGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAGTTACAGAC
CAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTTCACCGCTA
CACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCCAATGACCC
TCCCCGGTTGAGCCGGGGGGCTTTCACATCAGACTTAAGAAACCGCCTGCGAGCC
CTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACCGCGGCTGC
TGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGCCGCCCTAT
TTGAACGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAAAACCTTCA
TCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGATTCCCTACT
GCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGATCACCC
TCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACCAACTAGCT
AATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTATGTCTGAA
CCATGCGGTTCAAACAACCATCCGGTATTAGCCCCGGTTTCCCGGAGTTATCCCA
GTCTTACAGGCAGGTTACCCACGTGTTACTCACCCGTCGCCGC

16s rDNA SEQUENCES OF SEQUENCES OF *Bacillus velezensis*
CCGCGAATTACTAGCGATTCCAGCTTCACGCAGTCGAGTTGCAGACTGCGATCCG
AACTGAGAACAGATTTGTGGGATTGGCTTAACCTCGCGGTTTCGCTGCCCTTTGTT
CTGTCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCATGATGATTTGACG
TCATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTAGAGTGCCCAACTGA
ATGCTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCT
CACGACACGAGCTGACGACAACCATGCACCACCTGTCACTCTGCCCCCGAAGGG
GACGTCCTATCTCTAGGATTGTCAGAGGATGTCAAGACCTGGTAAGGTTCTTCGC
GTTGCTTCGAATTAAACCACATGCTCCACCGCTTGTGCGGGCCCCCGTCAATTCCT
TTGAGTTTCAGTCTTGCGACCGTACTCCCCAGGCGGAGTGCTTAATGCGTTAGCT
GCAGCACTAAGGGGCGGAAACCCCCTAACACTTAGCACTCATCGTTTACGGCGTG
GACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCAG
TTACAGACCAGAGAGTCGCCTTCGCCACTGGTGTTCCTCCACATCTCTACGCATTT
CACCGCTACACGTGGAATTCCACTCTCCTCTTCTGCACTCAAGTTCCCCAGTTTCC
AATGACCCTCCCCGGTTGAGCCGGGGGGCTTTCACATCAGACTTAAGAAACCGCC
TGCGAGCCCTTTACGCCCAATAATTCCGGACAACGCTTGCCACCTACGTATTACC
GCGGCTGCTGGCACGTAGTTAGCCGTGGCTTTCTGGTTAGGTACCGTCAAGGTGC
CGCCCTATTTGAACGGCACTTGTTCTTCCCTAACAACAGAGCTTTACGATCCGAA
AACCTTCATCACTCACGCGGCGTTGCTCCGTCAGACTTTCGTCCATTGCGGAAGA
TTCCCTACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCC
GATCACCCTCTCAGGTCGGCTACGCATCGTTGCCTTGGTGAGCCGTTACCTCACC
AACTAGCTAATGCGCCGCGGGTCCATCTGTAAGTGGTAGCCGAAGCCACCTTTTA
TGTCTGAACCATGCGGTTCAAACAACCATCCGGTATTAG

CCCCGGTTTCCCGGAGTTATCCCAGTCTTACAGGCAGGTTACCCACGTGTTACTCA
CCCGTCCGCCGC

ITS SEQUENCES OF *Candida tropicalis*

GAGGTACTCCGTAGGTGACCTGCGGCATATCAATAAGCGGAGGAACATCCGTAG
GTGAACCTGCGGCATATCAGTAATCGGAGGAACATCCTTATTATTTACTGTCAAA
CTTGATTTATTATTACAATAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTCGC
ATCGATGAAGAACGCAGCGAAATGCGATACGTAATATGAATTGCAGATATTCGT
GAATCATCGAATCTTTGAACGCACATTGCGCCCTTTGGTATTCCAAAGGGCATGC
CTGTTTGAGCGTCATTTCTCCCTCAAACCCCCGGGTTTGGTGTTGAGCAATACGCT
AGGTTTGTTTGAAAGAATTTAACGTGGAAACTTATTTTAAGCGACTTAGGTTTATC
CAAAAACCTTTATTTTGCTATTGGCCCCCACATTTTTTTTCAAAATTTTGCCCTCA
AATCGGGAAGAACTACCCCTTGAATTTAACCTTTTCAATAGCCGAAGAAAAGGGT
CCCCTCGGGGTGGAGGAGGTTCCCCA

PHYLLOSPHERE MICROORGANISMS FOR THE MANAGEMENT OF ANTHRACNOSE DISEASE OF COWPEA (*Vigna unguiculata*(L.) Walp.).

By

ANUPAMA P. R.

(2019-11-108)

ABSTRACT OF A THESIS

Submitted in partial fulfilment of the requirement for the degree of

Master of Science in Agriculture

(PLANT PATHOLOGY)

Faculty of Agriculture

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DEPARTMENT OF PLANT PATHOLOGY

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ABSTRACT

Microorganisms associated with aerial parts of plants such as stems (cauloshere), phylloplane (leaves), anthosphere (flowers) and carposphere (fruits) which are collectively known as phyllosphere. Phyllosphere microbes includes bacteria, filamentous fungi and yeasts. They offer natural biological control of diseases affecting aerial plant parts. Being better adapted to the phyllosphere niche, they are potential biocontrol agents which are under exploited in agriculture which allow them to adapt to the phyllosphere environment and inhibit the growth of microbial pathogens, thus sustaining plant health. Hence, microbial biocontrolbyphyllosphere microorganisms is a fast growing field of research. Considering the importance of the potential of phyllosphere antagonists, the research project entitled "Phyllosphere microorganisms for the management of anthracnose disease of cowpea (*Vigna unguiculata* (L.) Walp.)." was carried out. Phyllosphere microbiome enhance plant tolerance to withstand biotic and abiotic stress conditions in the current facet of climate change.

A purposive sampling survey was conducted in 21 locations of Kerala where cowpea and/or yard long bean are grown as a vegetable crop. The survey covered six districts namely, Thrissur, Palakkad, Malappuram, Kozhikode, Kannur and Kasaragod and five agro ecological units. Fungi associated with anthracnose symptoms and phylloshere microrganisms associated with healthy and symptomless plants from the fields were isolated. On pathogenicity test, six fungi were confirmed as pathogens and they were identified as *Colletotrichum Siamense*, three isolates of *Colletotrichum* sp., *Ectophoma multirostrata* and *Curvularia verruculosa* based on cultural, morphological and molecular characterization.*C. siamense* and *Ectophoma multirostrata* infecting cowpea is the first report in the world. Similarly, first report of *Curvularia verruculosa* infecting cowpea and producing anthracnose symptoms from India.

Enumeration of phyllosphere microorganisms on cowpea grown at different locations showed that population of fungi distributed on all plant parts and more abundant on flowers. Likewise, bacteria are more abundant on cowpea flowers, and fluorescent pseudomonads and yeasts are more abundantly seen on cowpea pods.

Based on colony characters, a total of 183 different isolates of phyllosphere microbes were made into pure cultures and were screened for antagonism towards *C. siamense*. Forty one isolates expressed antagonism in varying degrees which were again subjected to dual culture screening *in vitro*. Five promising anatgonists were selected based on per cent inhibition of mycelium of the pathogen on PDA. The five were identified as *Trichoderma longibrachiatum*, *Trichoderma asperellem*, *Bacillus amyloliquefaciens*, *Bacillus velezensis* and *Candida tropicalis* based on cultural, morphological and molecular characterization. Cell free culture filtrates of promising phyllosphere antagonists showed maximum growth inhitbion of *C. siamense* ranging from 86 per cent for *Candida tropicalis* to 100 per cent for *Trichoderma longibrachiatum* and *Trichoderma asperellem*

In order to study the induction of systemic resistance in cowpea by phyllosphere antagonists, a pot culture experiment was conducted and the results showed that all the antagonists accelerate the production of defence related enzymes such as peroxidase, polyphenol oxidase and phenylalanine ammonia lyase when challenge inoculated with the pathogen. *Bacillus amyloliquefaciens* induced higher activity of peroxidase (1.2 min-1g-1 fresh tissue over control), similarly polyphenol oxidase by *Candida tropicalis* (0.5min-1g-1 fresh tissue over control) and phenylalanine alanine by *Trichoderma asperellem* (0.8 µmol of transcinnamic acid formed g^{-1} fresh tissue over the control).

To test the biocontrol efficacy and plant growth promotion of phyllosphere antagonists against *C. siamense* causing cowpea anthracnose, a field experiment was conducted. The semi trailing cowpea variety 'Anashwara' was used in the experiment. The treatments included conventional biocontrol agents such as *Trichoderma* sp. (KAU) and *Pseudomonas fluorescens* (KAU) and chemical check with hexaconazole (systemic), mancozeb (contact) and carbendazim + mancozeb (systemic +contact) and a control without treatments were also included along with five phyllsophere antagonists such as *T. longibrachiatum*, *T. asperellem*, *B. amyloliquefaciens*, *B. velezensis* and *C. tropicalis.* All the treatments were given as seed treatment, and foliar spray. Higher vigour index of seedlings was recorded in the treatment with *Bacillus amyloliquefaciens* (1408). Biometric observations such as plant height, number of main branches, number of pods per plant, number of seeds per pod, fresh weight of pods,

pod length, days to first harvest, days to last harvest and total yieldand per cent disease incidence and severity were recorded at regular interval. Results of the field experiment indicated significant reduction in disease incidence and severity effected by phyllosphere antagonists such as *T. asperellem, B. amyloliquefaciens, B. velezensis, C. tropicalis, Trcihoderma* sp. (KAU), Pseudomonas fluorescens, hexaconazole, mancozeb and carbendazim + Mancozeb. In terms of yield all the five antagonists along with *Trichoderma* sp. (KAU) recorded significant yield increase in cowpea compared to other treatments.

The bacteria, *B. amyloliquefaciens* has gained more attention and an emerging evidence has identified its health beneficial effects as probiotics, hence farmers can safely use this as a biocontrol agent. Focusing on efficient and safer (non pathogenic nature), the phyllosphere antagonists such as *T. asperellem* and *B. amyloliquefaciens* are selected as the best among all the treatments. So, it can be concluded that out of the eleven treatments, *T. asperellem* and *B. amyloliquefaciens* could promote the growth of the plants and reduce the disease incidence and severity than the other treatments. However, multilocational field studies should be conducted to validate the findings of the current study, so that these bioagents can be formulated and made available to the farming community.