Investigations on Antagonism of Phylloplane Micro-flora of Mango against Mango Anthracnose.

By

Ms. JEETU NARWARE

B. Sc. (Ag.)

DEPARTMENT OF PLANT PATHOLOGY,

FACULTY OF AGRICULTURE,

DR. BALASAHEB SAWANT KONKAN KRISHI VIDYAPEETH,

DAPOLI - 415 712, DIST. RATNAGIRI (M.S.)

MAY, 2017

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(AGRICULTURAL UNIVERSITY)

DIST. RATNAGIRI (MAHARASHTRA STATE)

In partial fulfillment of the requirements for the degree of

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Approved by the Advisory Committee

: **Chairman and Research Guide :**

(P.G BORKAR)
Assistant Professor,
Department of Plant Pathology,
College of Agriculture, Dapoli.

: Members :

(M. S. JOSHI)
Professor (CAS),
Department of Plant Pathology,
College of Agriculture, Dapoli.

(M. S. KARMARKAR)
Assistant Professor,
Department of Agril. Entomology,
College of Agriculture, Dapoli.

(C. D. PAWAR)
Associate Professor,
Department of Horticulture,
College of Agriculture, Dapoli.

Dr. P. G. BORKAR
M.Sc. (Agri.), Ph.D.
Assistant Professor,
Department of Plant Pathology,
College of Agriculture, Dapoli.
Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth,
Dapoli – 415 712, Dist. Ratnagiri (M.S.)

CERTIFICATE

This is to certify that the thesis entitled, **"Investigations on antagonism of phylloplane micro-flora of mango against mango anthracnose"** submitted to the Faculty of Agriculture, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist. Ratnagiri, Maharashtra State, in the partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (Agriculture)** in **PLANT PATHOLOGY,** embodies the results of a piece of bona-fide research carried out by **Miss. JEETU NARWARE** under my guidance and supervision and that no part of this thesis has been submitted for any other degree or diploma or published in other form. All the assistance and help received during the course of investigation and the sources of literature have been duly acknowledged by her.

Place: Dapoli

Date : May 2017

(P.G. BORKAR)
Chairman,
Advisory Committee
and
Research Guide

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE, DAPOLI

Title of the Thesis	:	"Investigations on antagonism of Phylloplane micro-flora of mango against mango anthracnose"
Name of the student	:	*Miss. Jeetu Narware*
Regd. No.	:	2414
Name and Designation of the Research Guide	:	**Dr. P.G.Borkar** Assistant Professor, Department of Plant Pathology, College of Agriculture, Dapoli- 415 712.
Year of Award of Degree	:	2017

THESIS ABSTRACT

The present study entitled "Investigations on antagonism of Phylloplane micro-flora of mango against mango anthracnose" was carried out in the Department of Plant Pathology, College of Agriculture, Dr. BSKKV Dapoli, during 2016 -2017.

The pathogenic fungus was isolated on potato dextrose agar medium from anthracnose infected mango leaf. The pathogen was identified as *Colletotrichum gloeosporioides* (penz.) Penz.

All the fungicides tested against the pathogen *viz.,* Hexaconazole 25 EC (0.05%), Carbendiazim (0.1%), Sulphur (0.2%).

In vitro studies revealed that all the three fungal antagonists were effective against the pathogen but maximum per cent inhibition (72%) was recorded in *Aspergillus.* It was revealed that, the antagonist *Nigrospora sphaerica* (95. 56%) and *Aspergillus spp***.** (91.11%) *were* most compatible with sulphur *whereas Gliocladium roseum,* was more compatible with Hexaconazol (73.11 %).

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*I bow my head and knee with reverence before beloved father**Mr. KishanlalNarware**mother **Mrs. Kamal Narware**,my brother **Rahul Narware** and bhabhiSavitree,sister **MonikaNarware**,Raj Kishore Narware, sister sarojsuriyavanshi.Also I would like to thanks my cute Setukrut,Divyansh,Bhoomika,Isha,Punit.Any vocabulary will be insufficient to express my deepest sense of respect and gratitude towards my parents for their affection, words of encouragement, boundless love, unflagging inspiration, interest and selfless sacrifice.My relatives, who have been unending source of inspiration and warm affection for me.No words could justice to express my deepest sense of love and gratitude towards my friend and classmatessumaiyya, Nilesh, Pravin, Suraj, Yogini,Meenaandmy junior Komal,Anirudhha,Yogesh,Divyafor their excellent company, inspiration, moral support, boost up and healthy friendship.*

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Place: Dapoli *(Ms.JeetuNarware)*

Date: May, 2017

APPENDIX – I

ABBREVIATION'S USED

%	Per cent
/	Per
Spp.	Species
^{0}C	Degree celsius
C.D.	Critical Difference
cm	Centimeter
M	Molar
Conc.	Concentration
d.f.	Degree of freedom
Dist.	District
Dr.B.S.K.K.V.	Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth
E.M.S.	Error mean sum of square
et al.	And others
etc.	Etcetera
Fig.	Figure
g	Gram
µ	Micro gram
i.e.	That is
ml	micro litre
m	Meter
M.S.S.	Mean sum of square
mg	Milligram
L	Litre
P.D.A.	Potato Dextrose Agar
S.E.	Standard error
viz.	Namely
Sig.	Significant`
RHRelative Humidity	
@	At the rate

APPENDIX – II

ANOVA of (phylloplane fungi x*C. gloeosporioides*)

Source of variation	Degree of freedom	Sum of square	Mean sum of square	F-cal	F-tab	Result
Treatment	3	107.9975	35.99917	3348.76	5.010287	SIG
Error	16	0.172	0.01075			
Total	19	108.170				
S.Em.±	**0.05**					
C.D at 1 %	**0.19**					

APPENDIX – III

ANOVA for Compatibility of the antagonists with different fungicides by poisoned food technique

(a) *Nigrosporaspharica*

Source of variation	Degree of freedom	Sum of square	Mean sum of square	F-cal	F-tab	Result
Treatment	3	143.334	47.778	665.8955	5.010287	SIG
Error	16	1.148	0.07175			
Total	19	144.482				
S.Em.±	0.12					
C.D at 1 %	0.49					

(b) *Gliocladiumroseum*

Source of variation	Degree of freedom	Sum of square	Mean sum of square	F-cal	F-tab	Result
Treatment	3	171.778	57.25933	702.5685	5.010287	SIG
Error	16	1.304	0.0815			
Total	19	173.082				
S.Em.±	0.13					
C.D at 1 %	0.53					

(c) *Aspergillus spp.*

Source of variation	Degree of freedom	Sum of square	Mean sum of square	F-cal	F-tab	Result
Treatment	3	17.75	5.916667	118.3333	5.010287	SIG
Error	16	0.8	0.05			
Total	19	18.550				
S.Em.±	0.10					
C.D at 1 %	0.41					

APPENDIX – IV

LABORATORY MEDIA USED

Potato Dextrose Agar (PDA) Medium

i. Peeled potato : 200.00 g
ii. Dextrose : 20.00 g
iii. Agar- agar : 20.00 g
iv. Distilled water : 1000 ml

CHAPTER I

INTRODUCTION

Mango *(Mangiferaindica L.)* is known as “king of fruits” grown throughout the tropics and subtropics worldwide. India is the world’s largest producer of mango. The total annual world production of mango is 43,300,000 tonnes. (Anon.,s 2015 d). It is commercially grown in more than 111 countries, but nowhere it is as greatly valued as in India where 40 per cent of area under fruit crops is only under mango. India is the major mango producer in the world, with an area of 2.218 million hectares and the annual production of 18.832 million tones with productivity of 8.49 MT/ha. (Anon., 2015a). India contributes about 64 per cent of the world mango production. According to the APEDA, in the year 2014-15 India exported 42,998 tons of mangoes worth Rs.302 crores. (Anon.2015b).

According to recent statistical data, in Maharashtra, area covered during year 2014-15 was 0.156 million hectares with a production of 0.876 million tones and productivity of 5.60 mt/ha (Anon., 2015 c). In *Konkan*, 1, 82,000 ha area is under mango cultivation having annual production of 3.25 lakh MT. The productivity of mango in *Konkan* is about 2.5 t/ha, which is about three times less than the average productivity of the country (Anon., 2014). The *Konkan* region of Maharashtra state comprising of Palghar, Thane, Raigad, Ratnagiri and Sindhudurg districts are famous for the quality mangoes. About 90 per cent area under mango in the region is occupied by a single cultivar “Alphonso”, which is locally called as “*Hapus*”. It thrives and yields best under warm and humid climate of the region and is the best variety for table and processing purpose.

Diseases not only reduce yield but also greatly impair the quality and stability of production which consequently affect the agricultural sustainability. Mango is affected by a number of diseases. Amongthese, diseases caused by fungal pathogens are responsible for major crop losses. The most important diseases of Mango which limit its production are listed below.

Anthracnose	:	*Colletotrichumgloeosporioides(penz)*
Powdery mildew	:	*Oidiummangiferae*
Stem-end rots	:	*Lasidiplodiatheobromae(Botryodiplodiatheobromae)*
Blight	:	*Pestalotiamangiferae,Phyllosticta*spp.,*Alternaria*spp. *etc.*
Fruit rot	:	*C.gloesporioides,Aspergillus*spp., *Alternaria*spp.*etc.*

Wet, humid, warmweather condition favors anthracnose infection in the field. Spores (condia) of the pathogen are passively dispersed by rain splashes or wind currents. The pathogen perpetuates on infected and defoliated branch terminals and mature leaves. Anthracnose symptoms occuron leaves,twigs,petioles,panicles and fruits.Under field conditions,the initial appearance of the disease is manifested in the form of minute,sunken brown lesions on the tender foliage which enlarge into conspicuous brown spots within a week or so. Sometimes, the infected area of the leaf lamina is shed off giving a shot hole appearance. The sunken brown necrotic lesions retained on the leaf lamina then cover maximum leaf area. When the initial inoculum pressure is high, the infection usually spreads to the tender branches resulting in blossom blight. On fruits, the infection is evident in form of slightly depressed dark brown spots. However,this pathogen has quiescent mode of infection where in the fruits are

apparently healthy when green and start rotting rapidly upon ripening. This resultin tremendouspost-harvest losses during storage, transit and marketing.

Though, The disease can effectively controlled by an array of chemical fungicides available in the market, the long term effects of the residual toxicity of these chemicals is a matter great concern. Withthese view in mind, it was thought to take up initial work on phylloplane micro-flora of mango which may in turn, be the most tactical way for eco-friendly management of the disease in future.

The surface of aerial plant part provides a habitat for epiphytic micro-organism,many of which are capable of influencing the growth of pathogens.Phylloplane is the leaf surface which serves as a habitat for a variety of microorganisms including pathogens and saprophytes.The term phylloplane was given by 'Kerling' in 1964.Kerling(1964) used the term 'phylloplane' while referring to the actual leaf surface and 'phyllosphere' tothe zone nearleaves.

A number of saprophytic microorganisms on the phylloplane, antagonistic to pathogenhave been reported to produce antibiotics.

Considering the importance of above mentioned points the present investigation entitled "Investigation on antagonism of phylloplane micro-flora of mango against mango anthracnose" was undertaken with the following objectives:

1) Isolation and identification of phylloplane micro-flora of mango.
2) To test the antagonistic ability of isolated micro-flora against *C. gloeosporioidesin vitro*.
3) To test the compatibility of isolated phylloplanemicro-florawith recommendedfungicide*invitro*.

4) CHAPTERII

5) REVIEW OF LITERATURE

6) Members of the genus *Colletotrichum* are hemi-biotrophic Ascomycetes causing anthracnose on many crops plants. The genus was earlier placed in the family Melaconiaceae of the order Melaconiales but as per the recent classification of fungi, it is placed in the family Phyllachoraceae of the order Phyllachorales belonging to the class Sordariomycetes of phylum Ascomycota. The species of this genus such as *C. gloeosporioides*, *C. capsici*, *C. falcatum*,*C. truncatum*etc have been reported on a variety crops ranging from fruit crops, vegetables, ornamentals, agronomical crops and wild hosts including grasses (Gautam, 2014). Among all the reported species, *Colletotrichumgloeosporioides* is the most common and the looming plant pathogen. It is a widespread pathogen in diverse agro climatic conditions but devastating in hot and humid conditions. Mango anthracnose is the most serious disease of mango resulting in tremendous post harvest losses. The disease as well as the pathogen has been exhaustively studied and strategies for effective management of the disease have proposed by a number of research workers all over the globe. However, the work on phylloplane micro-flora of mango anthracnose is meager.

7) In this chapter, the literature on the disease, the pathogen, disease management strategies as well as the phylloplane micro-flora of the disease, collected from all possible sources, was reviewed and presented in the following pages.

8) 2.1. Disease report

9) Mango anthracnose wasreported for the first from Puerto Rico (Collins, 1903) and later from Hawaii.WhileMcRae (1924) reported it as causal organism of anthracnose of mango. McRae (1924) described the morphological characteristics of *Colletotrichumgloeosporioides*.

10) The post harvest fruit rot is the most damaging stage as it may result in economical losses to the tune of 15-20 per cent (Ploetz and Prakash, 1997). This phase is directly linked to the field phase where initial infections usually starts on tender leaves and later spreads to young twigs, flowers causing blossom blight, sometimes destroying the entire inflorescence and finally the pathogen may gain entry into the young fruits through stalk end and damage the fruits after harvest. *C.gloeosporioides*colonises dead twigs and injured plant tissues and forms abundant conidia in acervuli.

11) Ameera*et al.* (2010) observed that among the post harvest diseases anthracnose is the most important disease and it mostly prevalent in humid growing area, the incidence of this disease can reach almost 100 per cent under wet or very humid condition. Also found that postharvest losses of mango in tropical countries range from 15 percent in dry season and 70 percent in the rainy season.

12) Wang *et al.* (2016) reported that, in typical anthracnose symptoms, the lesions expand rapidly to cover the entire foliar surface, leading to defoliation of the tree at appropriate temperature and high humidity.

13) **2.2. Isolation of causal organism**

14) Soytong*et al.*(2005) obtained the isolates of *C. gloeosporioides*from leaves, twigs and fruitsof grape, showing anthracnose symptoms in vineyards all isolates were tested for pathogenicity to grape using Koch's Postulates.

15) Evueh and Ogbebor(2008) isolated C. *gloeosporioides*from the leaves of rubber seedlings by cuttingthe bits of 1 x 1 cm across lesions which were then surfaced sterilizedin 0.1% of mercuric chloride solution for 1 min followed rinsing in five changes of sterile distilled water.

16) *C. gloeosporioides*was isolated from infected mango fruits collected from mango orchards by tissue segment method and purifiedby single spore isolation methodby Mathews *et al.* (2010). The culture was maintained on Potato dextrose agar (PDA) for further studies.

17) Ismet*et al.* (2012) also isolated the pathogen from the fruits and the culture was maintained on PDA for further studies.

18) In an experiment conducted by Ghosh and Chakraborty(2012), *C. gloeosporioides* was isolated from infected leaves,petioles,twigs,panicles, flower and fruits on PDA.

19) Kuberan*et al.* (2012) reported the isolation of *Glomerellacingulata* inciting brown blight of tea leavesdisease pathogen. The infected leaves were collected and washed gently in distilled water and then dried by placing them in between folds of filter papers.

The dried bits were inoculated on PDA for obtaining pure culture.

20) Nagalakshmi*et al.* (2012)isolated the pathogen from the mango leaves showing typical anthracnose symptoms by tissue segment method and the culture was purified by single spore isolation method.

21) In a survey conducted by Lima *et al.* (2013)mango fruitsshowing post-harvest anthracnose symptoms was collected and the pathogen was isolated from small pieces (4 to 5 mm) of necrotic tissues.

22) 2.3.Isolation of phylloplane micro-flora

23) Breeze and Dix (1981) observed that the population of mycelium forming phylloplanefungi was the highest during July to October. But the yeast population was the maximum during November to May.

24) The method of isolation of phylloplane micro-flora by leaf impressionmethod has been discussed in detail by Aneja (2003).In order to isolate these organisms, both the leaf surfaces, dorsal and ventral,need to be pressed against the solid culture medium.

25) Salvikova*et al.* (2007, 2009) isolated seven yeast species*viz.* *Aureobasidiumpullulans*, *Cryptococcus laurentii*, *Pichiaanomala*, *Metschnikowiapulcherrima*, *Saccharomyces sp.*, *Lachanceathermotoleran*sand *Rhodotorulaglutinis*from the ten tree species in Thialand.

26) Evueh*et al.*(2008) isolatedphylloplane fungi from healthy leaves of rubber plantby leaf washing technique. In all the cultures of 10 phylloplane fungi *viz.Trichoderma*, *Aspergillus*, *GliocladiumPleurothecium*,*Botrytis*,*Staphylotrichum*,*Tricho*

cladium,*Gonatorrhodiell*a and *Trichophyton*species were found to be associated with rubber leaves.

27) Dilution plate method was employed for isolation of phylloplane micro-flora by Joshi *et al.*(2008).The collected leaf samples were cut into small pieces and1 g of sample was mixed thoroughlyin 100ml of distilled sterilized water and flask was shaken vigorously for 30 minutes. The suspension thus obtained was then subjected to 10^{-4}dilution and plated on PDA fortified with antibiotic. A quantity of 10 ml of this suspension was transferred aseptically to conical flask containing 90 ml of distilled sterile water and shaken vigorously to get homogenous suspension. Then 5 ml of such suspension was aseptically transferred into sterilized Petri plates containing solidified medium. Rose Bengal Agar medium was used for isolation of phylloplane fungi while Nutrient Agar medium was used to isolate bacteria. The fungal plates were incubated at 25±1^{0}C while those of bacteria were incubated at 30±1^{0}C for 5 days.

28) Mandhare and Suryawanshi (2009)used a modified leaf washing technique to estimate phylloplane micro-flora of safflower. The leaf samples of safflowerseedling were collected from the field and leaf discs of 5mm diameter were cut with a sterile cork borer. One hundred such discs were placed in 250ml conical flask containing 100ml distilled sterile water and shaken thoroughly for 20 minutes to get a homogenous suspension. One ml suspension was pour into sterilized Petri plates with a pipette. The plates were poured with potato dextrose agar medium (for fungi) and nutrient agar medium (for

bacteria) and mixed thoroughly. The Petriplates were then incubated at room temperature (26±°C).

29) In all 96 potential phylloplane antagonists including leaf endophytes were isolated from the samples collected from mango orchards in different mango regions of AndhraPradesh by Basha*et al.*(2010). The culture of antagonists was obtained by leaf washing followed by serial dilution method. Out of the ten promising antagonists, four were fungi (*Trichoderma* spp.) and six were bacteria (not identified).

30) In an attempt to isolate phylloplane fungi from three plant species *Ocimum sanctum, Phyllanthusamarus*and *Azadirachtaindica*it was found that, *Aspergillusflavus*,*Penicilliumexpansum, Fusariumsemitectum*, *Fusariumoxysporum* were associated with the leaves *Ocimum sanctum*. *Scopulariopsis* spp. was associated with phylloplane of *Phyllanthusamarus*, while *Penicilliumjanthinellum*, *Aspergillusfumiculosis*, *Aspergillus* spp., *Curvularialunata* and *Fusariummoniliforme* were found on *Azadirachtaindica* (Prabhakaran *et al.*,2011).

31) Kuberan*et al.* (2012) isolatedseven species associated with tea phylloplane samples collected from different tea gardens of southern India by leaf washing technique on PDA medium.

32) Isolation and identification of antagonistic bacteria from phylloplane of rice to use as bio-control agents against sheath blight of rice was reported by Akter*et al.* (2013). Out of 325 isolates, 14 were found to be effective as antagonists of *Rhizhoctoniasolani*.

33) Deepika*et al.*(2014) isolated17 phylloplane fungi of *Buteamonosperma* from Uttarakhand, on Martin's agar medium. Among the 17 isolated cultures three each belonged to *Alternaria* and *Penicillium,* five to *Aspergillus,* two *Cladosporium*, one each of *Tricoderma,Fusarium, and Curvularia* and one fungal culture with white sterile mycelium was unidentified.

34) Ogwu *et al.*(2014)isolated8 fungi *viz. Saccharomyces*, *Rhodotorula*, *Mucor*, *Aspergillus,Pencilium*, *Tricodrerma,Cladosporium, Rhizopus*and *Botrydiplodia*and 6 bacteria from phylloplane of okra field in Nigeria.

35) Endophytic fungi of tea were isolated by Rabha*et al.* (2014).

36) Sahu*et al.* (2014) isolated phylloplane micro-flora of *Hibiscus sabdariffa*by using a cut leaf suspension poured on PDA.

37) *Alternariaaltarnata* causes leaf spot of *Spilanthusoleracea*.Shikha*et al.*(2014) isolated phylloplane fungi from healthy leaves of this plant by leaf washing technique to test their efficacy as antagonists against the pathogen. Out of the 10 isolates obtained 5 were species of Pennicillium, 3 were of *Trichoderma* and one each of *Aspergillus* and *Cladosporium*. The morpho-taxonomy of the fungi was confirmed with help of standard monographs. The pure cultures were maintained on PDA.

38) PhylloplaneYeastwas isolated from healthy leaves of geranium, vinca, dogwood, and henbit by Buck *et al.*(2002).

39) 2.4. Dual culture test for selection of antagonistic phylloplanemicro-flora

40) Basha*et al.*(2010) tested the antagonism of phylloplane micro-flora of mango against *C. gloeosporioides* by dual culture technique, in case of fungal antagonists, mycelia discs of 6mm diameter cut from seven day old culture of the antagonists and the pathogen were transferred to PDA medium and inhibition percentage was calculated on the basis of colony diameter. Culture of the bacterial antagonist was streaked against the culture bit of the pathogen to calculate the inhibition bythe bacterial antagonist.

41) Kuberan*et al.* (2012) also used dual culture technique to assess the antagonistic potential of phylloplane inhabiting *Trichoderma* against*G.cingulata.*

42) Interaction between the phylloplane fungi and the leaf spot pathogen of *Steriarebaudiana-Alternariaalternata* was studied by Chauhan and Navneet (2015). The results were categorizedinto 5 categories as 0- No visible sign of inhibition,1-Pathogenic fungi overgrew the test organism.2- Mutual inhibitions, both organisms stopped growing on contact, 3-Inhibition of pathogen with inhibition zone > 1cm in width, 4-Inhibition of pathogen with inhibition zone < 1cm in width, 5- Inhibition of pathogen by over growth of the antagonists.

43) 2.5.To test the compatibility of isolated phylloplane micro-flora with recommended fungicide *in vitro*

44) The *in vitro* sensitivity of the two groups of yeast isolates with nine fungicides was evaluated on fungicide amended PDA by Buck and Burpe (2002)Yeast suspension was poured into Petri dishes containing PDA amended with different concentrations of each fungicide *viz*. chloramphenicol, thiophanate-methyl, chlorothalonil, vinclozolin, iprodione, flutolanil, azoxystrobin, myclobutanil, propiconazole and. Mancozeb. Dishes ofnon amended PDA served as control. Growth was evaluated in a 0–3 scale (0, no growth; 1, individual colonies; 2, reduced and (or) slow growth; 3, growth on non amended PDA. Significant differences in growth between the two groups of yeast were observed. The results revealed that Thiobendazole at lower concentrations improved the efficacy of the yeast cultures under study.

45) Lima et *al.* (2008)studiedtheeffect offungicides on potential antagonists *in vitro* on liquid as well as solid media amended with various concentrations of the fungicides to be tested. Results of the resistance of BCAs *in vitro*, as an isolate of the biocontrol yeast *Rhodotorulaglutinis*(LS11), though highly sensitive to benzimidazoles*in vitro*, when applied in combination with benomyl on apples in semi-commercial conditions, was as effective as other *in vitro* fungicide-resistant isolates. Similarly, in a study of the compatibility of a BCA with some fungicides for the control of tomato diseases,chemicals were toxic to BCA *in vitro*, but less

toxic in greenhouse tests, probably because they were applied as sprays to the plant.

46) Basha*et al.* (2010) studied the compatibility of phylloplane*Trichoderma*isolates with six fungicides *viz.* carbendazimthiophanate–methyl,propioconazole,hexaconazole and two non-systemic fungicides *viz.*, mancozeb and copper oxy-chloride. The result revealed that mancozeb was the most compatible fungicide.

47) Nagalakshmi*et al.* (2012)found that from 21 potential bio-control agents ,bacterial isolate - BL5 (leaf endophyte) was the most compatible with thiophanate–methyl (100%) followed by mancozeb (98.33%) as compared to the remaining four fungicides.

48)

CHAPTER III

MATERIALS AND METHODS

The various experiments in the present study on use of phylloplane micro-flora of mango against mango anthracnose were carried out in the Department of Plant Pathology, College of Agriculture, Dr. B.S.K.K.V., Dapoli, during 2016 -2017.

The materials used and methods or techniques adopted during the course of present investigation are mentioned below.

Materials:

The following materials were used during the course of the present studies.

Disease sample:

The infected leaf samples showing typical symptoms of anthracnose of mango were collected in paper bags from the unsprayed, naturally maintained mango orchard of the department of Horticulture, of Dr. B.S.K.K.V., Dapoli, and brought to the laboratory for further studies.

Culture Media:

Routinely used laboratory medium for isolation of fungi *i.e.* Potato Dextrose Agar (PDA) medium was used for isolation of the causal organism from infected leaf samples.

Chemicals:

The chemicals used for *in vitro* and *in vivo* experiments were of analytical grade and were obtained from Department of Plant Pathology, Dr. B.S.K.K.V., Dapoli.

Glassware:

Standard Borosil and corning brand glassware was used during the course of research work.

Equipment's:

Common laboratory equipments such as, autoclave, laminar air flow bench, incubator, refrigerator, burner, plastic trays and electronic top pan balance were used for lab work.

Chemicals and other material:

The fungicides such as, Sulphur (Sulpho), Hexaconazole (Topper), and Carbendazim (Bavistin) were used during present studies. All the fungicides used for the investigation were from fresh stock.

Mango seedlings:

Mango seedlings used in the present studies were obtained from the nursery of horticulture department.

Miscellaneous materials:

Cork borer, polythene bags, forceps, inoculation needle, gas burner, cotton etc were used during the course of investigation.

Methods:

3.1. Examination of disease samples:

3.1.1. Visual observation:

Visual observations on symptoms were recorded in field to know the development of the disease in a plant population under natural conditions.

3.1.2. Microscopic examination:

Fresh disease sample showing typical symptoms of anthracnose were collected and brought to the laboratory. These samples were then washed under tap water to remove extraneous material. Temporary mounts prepared from the diseased specimens in lacto phenol cotton blue and examined under microscope.

3.1.3. Isolation of causal organism:

Collected sample were washed with running tap water to remove extraneous material. Small bits of desired size were cut by taking care that each bit contained half infected and half healthy portion. Such bits were then disinfected with 1 per cent sodium hypochlorite solution for 30 seconds followed by three washings in distilled sterile water to remove the traces of sodium hypochlorite. These bits were then placed on sterilized blotters for drying. Properly dried bits were transferred aseptically in sterilized Petri plates containing sterilized, solidified PDA medium. The plates were incubated in BOD incubator at 26±1°C till the fungal mycelium fully covered the surface of the medium. The bits of fully developed fungal growth were cut with sterilized cork borer and transferred to PDA slants and maintained as stock culture for further studies. The culture was maintained by periodical transfer.

3.2. Isolation and identification of phylloplane micro-flora:

3.2.1. Isolation:

The tender, healthy leaves of mango were collected from the mango orchard in paper bags and brought to the laboratory. PDA was prepared by standard procedure and poured in sterilized Petri plates in laminar air flow chamber. Each leaf sample was pressed from dorsal as well as ventral surface separately against the sterilized semi liquid agar medium. The procedure was repeated twice to confirm whether specific phylloplane micro-flora is constantly associated with mango leaves. The plates were incubated at 26 ± 1^0 C for seven days. After seven days, individual isolates with profuse mycelial growth were selected and were transferred to PDA in sterilized Petri plates. Pure cultures were maintained by periodic transfer PDA slants. Spores are the unique feature of each fungus which confirms their morphological identification. Some fungi sporulate easily in culture while others require specific treatment. Light is essential for sporulation of most of the fungi but some fungi sporulate better in diffused light while others sporulate in alternate light and dark conditions. In order to confirm sporulation of the isolated fugal antagonists, the culture plates were exposed to continuous light, continuous darkness and alternate light and dark conditions for a week period. The cultures were observed under microscope for confirmation after 7 days.

3.2.2. Identification:

The pure cultures of the phylloplane micro-flora obtained on isolation were observed under microscope by preparing temporary mounts. The cultures were tentatively identified by comparing morphological and colony characters with the information available in the reviewed literature as well as on the standard websites for fungal identification. Two cultures of the isolated unknown fungi were sent to the Chief Mycologist, Agharkar Research Institute, Pune, for confirmation up to species level.

3.3. Testing the antagonistic ability of potential phylloplane antagonists against *C. gloeosporioides* by dual culture technique:

The antagonistic ability of the pure isolates against *C. gloeosporioides* was determined by dual culture technique under *in vitro* conditions. Mycelial discs of 6 mm diameter of seven day old cultures of the isolated fungal antagonists and *C. gloeosporioides* were placed equidistantly from the centre of Petri plates containing PDA medium. The Petri plates were then incubated at 26 ± 1^0 C. Three replications were maintained in each treatment. The plates with mycelial bits of the pathogen alone served as control. The observations on growth of both the organisms were recorded on 7^{th} day after inoculation. Inhibition percentage of mycelial growth of the pathogen was calculated by the formula:

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

Where,

I = Per cent inhibition

C = Radial growth (cm) in control

T = Radial growth (cm) in treatment. (Denis and Webster, 1971).

In order to record the antagonistic effect in dual culture, the total colony area of isolated phylloplane micro-flora as well as the pathogen (C. *gloeosporioides)* was plotted on graph paper to measure per cent inhibition by phylloplane micro-flora.

On confirming the antagonistic activity of phylloplane micro-flora against the *C. gloeosporioides*, the cultures were identified by comparing morphological and colony characters with the information available in the reviewed literature as well as on the standard websites for fungal identification .Two cultures of the isolated unknown fungi were sent to the Chief Mycologist, Agharkar Research Institute, Pune, for confirmation up to species level while the culture of *Aspergillus* was identified up to genus level.

3.4. Compatibility of potential antagonists with different fungicides.

This experiment was conducted to test the compatibility of potential antagonists of *C. gloeosporioides* with the fungicides recommended against the pathogen. Two systemic fungicides *viz.*, carbendazim (0.1% -1 g/L), hexaconazole (0.05 % -1/2 ml/L) and one non-systemic fungicides *viz.*, Sulphur (0.2% - 2g /L) were evaluated for their compatibility with potential antagonists by poisoned food technique (Nene and Thapliyal (1997).

Tr. No.	Treatments	Trade name	Conc. (%)
T_1	Sulphur	Sulpho	0.2 %
T_2	Hexaconazole 25 EC	Topper	0.05%
T_3	Carbendiazim	Bavistin	0.1%
T_4	Control	-	-

Fungicidal solution of required concentration was prepared and it was poured in to 100 ml PDA in measured quantity to get the desired concentration. Poisoned medium (15 ml) was poured in sterile Petri plates and allowed to solidify. A 5 mm mycelial disc of seven days old culture of each antagonist was inoculated separately at the centre of each Petri plate and incubated at 26±1°C and

maintained for ten days. A control was maintained without fungicide. Three replications were maintained per treatment. Per cent reduction in radial growth was compared with growth in control plates and per cent compatibility was calculated by the following formula:

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

Where,

I = Per cent compatibility.

C = Radial growth (cm) in control.

T = Radial growth (cm) in treatment. (Denis and Webster, 1971).

The total colony area in all the treatments was plotted on graph paper as mentioned earlier.

3.5. Evaluation of isolated phylloplane micro-flora against *C. gloeosporioides in vivo* :

Evaluation of the antagonistic ability of phylloplane micro-flora under field conditions is possible by spraying the suspension of the organism on host surface. In case of fungal antagonists, it is necessary to spray the mycelial suspension containing spores as the spores have inherent ability to adhere, establish and germinate on the host. In order to get a suspension, the cultures of all the antagonists were transferred to potato dextrose broth in flasks and incubated at ambient temperature up to 7 days. When a thick mycelial mat of the antagonists was formed on broth medium, it was strained through muslin cloth to harvest the mycelium. The harvested mycelial mat along with spores was ground separately in

a kitchen grinder by adding required quantity of distilled sterile water in order to avoid chocking of spray nozzle. The mycelial suspension thus obtained was used for spraying. Disease free, healthy, 25 days old mango seedlings with coppery red foliage were used for spraying mycelial suspension. The leaves of the seedlings were washed with distilled sterile water to ensure the elimination of any other phylloplane organism as well as dust particles if any. When the foliage of the seedlings was air dried, the suspension of each antagonist was sprayed separately on the seedlings. Five seedlings were maintained for suspension of each antagonist. The seedlings were then transferred to a muslin cloth moist chamber to provide required humidity. The antagonists were allowed to establish on the foliage for a period of 7 days. Required humidity was maintained by frequently moistening the muslin cloth with water. The spore suspension of *C. gloeosporioides* was sprayed with an atomizer on each treated seedling. Total leaf area was calculated by leaf area meter and per cent disease incidence was recorded by plotting infected area on graph paper.

3.6 Statistical Analysis

The data obtained in all experiments were statistically analyzed using methods Completely Randomized Design (CRD) was used for dual culture and FCRD for poisoned food technique.

CHAPTER IV

EXPERIMENTAL RESULTS

Phylloplane micro-flora comprises a group of different microbes such as bacteria, mycelium forming fungi, yeasts etc. which are the inhabitants of the plant foliage. The present study was conducted to isolate such microbes and to assess their potential to perform as antagonists against the mango anthracnose fungus *C. gloeosporioides*. The results of the experiments carried out in this regard are presented in this chapter.

4.1. Examination of diseased samples:

4.1.1. Visual observations:

The leaf samples collected from the naturally maintained, unsprayed mango orchard revealed the typical symptoms of anthracnose of mango leaves. The infection of the pathogen resulted in the formation of dark brown sunken spots with irregular margin.

4.1.2. Microscopic examination:

The microscopic examination of infected tissues revealed the presence of rod shaped spores with two oil globules. The asexual spore fruit of *C. gloeosporioides* -acervulus was also observed under microscope.

4.1.3. Isolation of causal organism:

The pathogen *C. gloeosporioides* grew profusely on PDA. The colony was white initially which gradually turned grey in colour. The mycelium completely covered the surface of the medium within 7 days. The bits of fully grown mycelium were cut with a sterile cork borer and transferred to PDA slants to use as stock culture for further studies.

4.2. Isolation and identification of phylloplane micro-flora:

4.2.1. Isolation:

Repeated isolations of phylloplane micro-flora of mango revealed the presence of three fungi. In present study, other phylloplane organisms such as bacteria and yeasts were not found to be associated with mango leaves. The colony of one of the isolated fungus was pink in colour. The growth of this fungus on PDA was very slow at ambient temperature. The colony of the second fungus was creamy white and slightly sticky. The third isolated fungus formed dark black colony on PDA and its growth was fast as it reached to the rim of the Petri plate within four days.

4.2.2. Identification:

In microscopic observations one of the three fungal antagonists was confirmed as *Aspergillus* on the basis of morphological characters such as septate mycelium, collumela formed in apical region of the conidiophores and round black coloured spores. The remaining two unidentified cultures were sent for identification to The Chief Mycologist, Agharkar Research Institute, Pune. The fungus forming pink colony was identified as *Gliocladium roseaum* Bainier and the fungus with creamy white mycelium was identified as *Nigrospora sphaerica* (Sacc.) E. W. Mason.

4.3. Testing the antagonistic ability of isolated phylloplane fungi against *C. gloeosporioides* by dual culture:

Table 1. Phylloplane fungi against x *C. gloeosporioides* by dual culture

Treatments	Mean colony diameter of the antagonist	Mean colony diameter of *C. gloeosporioides*	% inhibition
T_1-*Gliocladium*	6.56	2.44	27.11
T_2-*Nigrospora*	5.54	3.46	38.44
T_3-*Aspergillus*	6.48	2.52	72.00
T_4- control	9.00	9.00	-
SE (M)±	**0.05**		
CD @1%	**0.19**		

It is revealed from the data presented in Table 1 that all the three fungal antagonists were effective against the pathogen. Maximum per cent inhibition (72%) was recorded in *Aspergillus* followed by *Nigrospora* (38.44 %) and *Gliocladium* (27.11%).

4.4. Compatibility of the antagonists with different fungicides by poisoned food technique

Table 2. *Nigrospora spharica (Sacc.)* E.W.Mason

Treatments	Mean colony dia. (cm)	% inhibition	% compatibility
T_1 Sulphur	8.60	4.44	95.56
T_2 Hexaconazol	2.28	74.67	25.33
T_3 Carbendazim	7.20	20.00	80.00
T_4 Control	9.00		
SEm ±	**0.12**		
CD @1%	**0.49**		

The results in Table 2 indicate that the antagonist *Nigrospora spharica* was the most compatible with sulphur (95.56%) followed by Carbendazim (80.00%). Hexaconazole (25.33 %) was found to be slightly detrimental for the mycelial growth of the fungus.

Table 3. *Gliocladium roseum* Bainier

Treatments	**Mean colony dia. (cm)**	**% inhibition**	**% compatibility**
T_1 Sulphur	**3.72**	58.67	41.33
T_2 Hexaconazol	**6.58**	26.89	73.11
T_3 Carbendazim	**1.22**	86.44	13.56
T_4 Control	**9.00**		
S.Em±	**0.13**		
CD @1%	**0.53**		

Table 3 revealed that the antagonist was more compatible with Hexaconazole (73.11%) followed by sulphur (41.33%). carbendazim was found to be detrimental for the mycelial growth of the fungus.

Table 4. *Aspergillus spp.*

Treatments	**Mean colony dia. (cm)**	**% inhibition**	**% compatibility**
T_1 Sulphur	8.20	8.89	91.11
T_2 Hexaconazol	6.60	26.67	73.33
T_3 Carbendazim	8.80	2.22	97.78
T_4 Control	9.00		
SEm±	**0.10**		
CD @1%	**0.41**		

The results of Table 4 indicate that the antagonist was compatible with all the three fungicides but the most compatible with carbendazim (97.78 %) followed by sulphur (91.11%) and Hexaconazole (73.33%).

4.5. Evaluation of isolated phylloplane antagonists against *C. gloeosporioides in vivo*:

Table 5.

Treatments	**Total Mean healthy leaf area of the seedling (cm)**	**Total mean diseased leaf area of the seedling (cm)**	**% diseased area (cm)**
T_1 *Nigrospora*	96.40	14.00	14.52
T_2 *Gliocladium*	107.04	21.5	20.08
T_3 *Aspergillus*	115.33	25.6	22.19
T_4 Control	86.98	36.91	42.43

It is revealed from the data presented in Table 5 that *Nigrospora sphaerica* was the best phylloplane antagonist as application of its suspension on the foliage resulted in reduction of diseased area. It was followed by *Gliocladium* and *Aspergillus*.

CHAPTER V

DISCUSSION

Mango is the major fruit crop of world as well as India. It is cultivated mainly in tropical and subtropical parts of the country. Anthracnose of mango caused by *Colletotrichum gloeosporioides* is a well-established disease in many mango growing regions. Climatic condition of konkan region is suitable for cultivation of mango. These climatic conditions are also suitable for the growth, reproduction and dissemination of the pathogen inciting mango anthracnose. In humid tropics, severe incidence of anthracnose is common from seedling stage to post harvest stage. Considering the importance of the crop and the devastating incidence of the disease, it was thought essential to undertake the systematic studies on isolation of phylloplane micro-flora of mango and its probable relevance in management of mango anthracnose in an eco-friendly manner.

The anthracnose infected leaves were collected from the naturally maintained, unsprayed mango orchard and pathogen *C. gloeosporioides* was isolated on PDA. Soytong *et al.* (2005), Evueh and Ogbebor (2008), Ismet *et al.* (2012) and Kuberan *et.al* (2012), also reported that PDA is the best medium both for mycelial growth as well as spore formation of *Colletotrichum gloeosporioides.*

In the present study, isolation of phylloplane micro-flora was done by using leaf impression method where, both the leaf surfaces, dorsal and ventral, were pressed against the solid culture medium as per the method described by Aneja (2003), for isolation of phylloplane micro-flora.

The experiment on antagonistic effect of phylloplane fungi revealed that all the three isolated fungal antagonists inhibited the growth of *C. gloeosporioides* at varying degree in dual culture. Among the three phylloplane fungal antagonists, *Aspergillus* was the best with highest per cent of inhibition followed by *Nigrospora sphaerica* (Sacc.) E. W. Mason and *Gliocladium roseum.* It may either due to competition for space, due to secretion of volatile chemical which is toxic to the pathogen or due to hyper parasitism. Similar results were also reported by Mathews *et al.* (2010) who isolated *Trichoderma* from phylloplane of mango in Andhra Pradesh and it was found to be a potential antagonist against the anthracnose pathogen *in vitro*. Kuberan *et al* (2012) also reported similar results.

Antagonistic ability of *Gliocladium* spp and *Aspergillus* spp. against *C. gloeosporioides* was reported Evueh *et al.* (2008).They concluded that the antagonists coagulate the cytoplasmic contents of the pathogen which consequently results in lysis of apical tip of the mycelium of the pathogen and leads to its death. In the present study also *Gliocladium roseaum* and *Aspergillus* species were isolated from mango phylloplane and were effective as antagonists against C. *gloeosporioides*. Hence the results are in concurrence with those of Evueh *et al.* (2008).

Mathews *et al.* (2010) studied the compatibility of four phylloplane *Trichoderma* isolates used as antagonists against *C. gloeosporioides* with various fungicides at different concentrations. Among the four isolates, the isolates T_1 and T_7 were 100 per cent compatible with Mancozeb. The isolate T_7 was also compatible with Thiram but Thiram had inhibitory effect on T_1. The results of the present study are in similitude with them. The compatibility of three phylloplane antagonists with different fungicide was not similar. Out of the three antagonists two were (*Nigrospora spharica* and

Aspergillus spp.) more compatible with sulphur. It may be due to the fact that, sulphur plays vital role in growth and reproduction of many fungi and also acts as a component of sulphur containing amino acid in protein synthesis. The inhibitory effect of sulphur on *Gliocladium roseum* may be due to the difference chitin content and chitin synthesis process of this fungus.

In case of Hexaconazole, it was found that, *Aspergillus* and *Gliocladium roseum* were more compatible (above 73 %) while *Nigrospora spharica* was the least compatible (25.33 %).

In respect of Carbendazim, *Aspergillus* and *Nigrospora* were compatible with it to the tune of 97.78 per cent and 80 per cent but it was harmful for *Gliocladium roseum.* None of the authors in reviewed literature had worked on fungicidal compatibility of antagonists hence the results could not be compared.

The results of the experiment on application of mycelial suspension of the antagonists in the field revealed that, all the three antagonists were effective against the anthracnose pathogen. Among the three antagonists, *Nigrospora* was the most effective with 85.48 per cent control (14.52 % disease incidence) followed by *Gliocladium* (79.92 % control – 20.08 % disease incidence) and *Aspergillus* (77.81 % and 22.19 % disease incidence). Similar studies on use of antagonists were not conducted by earlier workers hence the results cannot be compared.

CHAPTER VI

SUMMARY AND CONCLUSION

The various experiments in the present study on investigations onphylloplane micro-flora of mango against mango anthracnose were carried out in the Department of Plant Pathology, College of Agriculture, Dr. B.S.K.K.V.,Dapoli, during 2016 -2017.

The pathogenic fungus was isolated on potato dextrose agar medium from anthracnose infected mango leaves. The pathogen was identified as *C. gloeosporioides* on the basis of morphological characters and cultural characteristics.

Isolation of phylloplanefungal antagonists was done by leaf impression method on semi liquid potato dextrose agar medium.

In the *in vitro* experiment of testing the antagonistic ability of isolated phylloplane fungi against *C. gloeosporioides*by dual cultureit was revealed that, all the three fungal antagonists were effective against the pathogen but maximum per cent inhibition (72%) was recorded in *Aspergillus*.

Compatibility of isolated fungal antagonists with three fungicides was assessed by poisoned food technique. In this it was revealed that, the antagonist *Nigrosporasphaerica*(95.56%) and *Aspergillus spp.*(91.11%) were most compatible with sulphurwhereas*Gliocladiumroseum,*was more compatible with Hexaconazol (73.11 %).

Study on the evaluation of isolated phylloplanefungal antagonists against *C. gloeosporioides*under *in vivo* condition, revealed that *Nigrosporasphaerica*was the best phylloplane antagonist.

The results of present study are quite encouraging for the eco-friendly management of the mango anthracnose but some more potential phylloplane fungal as well as bacterial antagonists may be present at different locations in the region. There is a need to isolate all such antagonists and study their interactions with each other to formulate consortium of synergistic microbes for the better management of the disease and thereby provide a pollution free technology for disease management to the farming community in *Konkan* region.

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