"STUDIES ON HEART ROT OF POMEGRANATE CAUSED BY *ALTERNARIA ALTERNATA* (FR.) KEISSL."

BY

SIRSAT JAYDIP DILIP

B.Sc. (Agriculture)

A thesis submitted to

Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani

in partial fulfillment of the requirements for the Degree of

MASTER OF SCIENCE

IN

AGRICULTURE

(PLANT PATHOLOGY)

DEPARTMENT OF PLANT PATHOLOGY

COLLEGE OF AGRICULTURE, PARBHANI

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PARBHANI 431 402 (M.S.) INDIA

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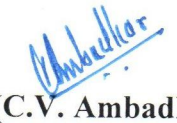

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Place: Parbhani

Date: ***(Mr. Sirsat Jaydip Dilip)***

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ABBREVIATIONS

/	-	Per
-	-	Minus
%	-	Per cent
@	-	At the rate of
µm	-	Micrometer
Avg.	-	Average
CRD	-	Completely Randomized Design
Conc.	-	Concentration (s)
C.D.	-	Critical difference
Cv.		Cultivar/Coefficient of variation
DAI	-	Days after inoculation
°C	-	Degree Celsius
Dia.	-	Diameter
e.g.,	-	Example Gratia (for example)
et al.,	-	and others
etc.	-	Etcetera
Fig.	-	Figure (s)
f. sp.	-	Forma Species
gm	-	Gram
hr	-	Hour (s)
i.e.	-	That is
Max.	-	Maximum
Min.	-	Minimum
ml	-	Millilitre
mm	-	Millimeter
no.	-	Number (s)
PDA	-	Potato Dextrose Agar
S.E.	-	Standard Error
spp.	-	Species
Tr. and T	-	Treatment (s)
viz.,	-	Videlicet (namely)

THESIS ABSTRACT

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1	Title of thesis	:	"Studies on heart rot of Pomegranate caused by *Alternaria alternata* (Fr.) Keissl."
2	Name of candidate	:	Sirsat Jaydip Dilip
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5	University	:	V.N.M.K.V., Parbhani
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ABSTRACT

Pomegranate is a finest gift from Iran to central and northern India which is considered as super food. Now a days the export quality of pomegranate is decreasing due to many post harvest diseases as well as the loss in storage is also worsening due to fruit rot and heart rot caused by several post harvest pathogens among which *Colletotrichum gleosporioides* and *Alternaria alternata* are being major one with losses accounting upto 15-80 per cent. So, meticulous investigation is carried out on "Studies on management of heart rot of Pomegranate caused by *Alternaria alternata*" during 2020-21 at Department of Plant Pathology, College of Agriculture, Parbhani and NRC on Pomegranate, Solapur.

During present investigation, the leaf spot and heart rot diseased plants specimens (Leaves and Fruits) were collected from fields of farmer various locations which were subjected to the isolation on PDA, where *Alternaria alternata* (Fr.) Keissl was isolated. The pathogenicity of these test pathogen was proved successfully in screen house on pomegranate seedlings by spray inoculation of spore suspension and in laboratory on fruits by pin prick and cotton swab method.

In the cultural and morphological studies of *Alternaria alternata*, it was observed that, Oat meal agar media was found most suitable for growth of *Alternaria alternata* followed by Malt extract agar media. Amongst all the tested media, the least growth of *Alternaria alternata* was recorded on Richard's agar media.

All the seven bioagents evaluated *in vitro* were found effective against *Alternaria alternata.* However, *Trichoderma asperellum* was promising which inhibited 81.48 per cent mycelial growth of *Alternaria alternata.*

In vitro efficacy of phytoextracts against *Alternaria alternata* were tested @ 10 and 20 per cent. Amongst the tested phytoextracts, Neem leaf extract at 10 and 20 per cent was found most significant with highest mycelial growth inhibition i.e 55.54 and 53.74 per cent, respectively

In vitro efficacy of essential oils against *Alternaria alternata* was tested at 2000 and 2500 ppm. Among them Citronella, Rose and Clove oils were found most effective with cent per cent mycelial growth inhibition at both concentrations

Besides this, different types of Ecolaid microbicides were also tested against *Alternaria alternata*. Among these all Ecolaid microbicides, the Ecolaid microbicides evaluated at 250 and 500 ppm were found most effective with cent per cent mycelial growth inhibition of test pathogen.

CHAPTER - I

INTRODUCTION

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INTRODUCTION

Pomegranate (*Punica granatum* L.), a tropical and subtropical fruit crop, belongs to the *Lythraceae* family and is an old and commercially important fruit crop. Pomegranates are native to Iran, where they were originally cultivated approximately 2000 BC, but they quickly migrated to Mediterranean countries. It is now widely grown in Spain, Morocco, and other Mediterranean countries, as well as Egypt, Iran, and Afghanistan. India leads the world in terms of acreage (262 million acres) and fruit production (3034 million tons) Maharashtra, known as India's pomegranate basket, contributes more than 70% of the entire pomegranate area, followed by Gujarat, Karnataka, Andhra Pradesh, Madhya Pradesh, Rajasthan, the major states commercially cultivating pomegranates on a vast scale. Pomegranate covers 147.91 thousand hectares in Maharashtra, with an annual production of 1789.46 thousand tons (Anon, 2018). Solapur, Sangli, Nashik, Ahmednagar, Pune, Dhule, Aurangabad, Satara, Osmanabad, and Latur are among the areas in Maharashtra where it is widely grown.

Pomegranate has both culinary and medicinal properties. It is most well-known for its pleasant juice. Pomegranates can also be found in cooking, juice blends, meal garnishes, and alcoholic drinks. Pomegranate fruits include 78 per cent moisture, 5.1 per cent fiber, 14.5 per cent carbs, 100 mg calcium, 70 mg phosphorous, and 16 percent vitamin C per 100-gram edible section. It has the ability to neutralize free radicals while also providing antioxidants, tannins, and anthocyanins. Sore throats, coughs, skin problems, and stomach problems have all been treated with it. Pomegranate may also be beneficial in the treatment of major illnesses such as cancer and diabetes. The seeds are dried and marketed as 'Anardana,' a spice (Bhowmik *et al*.,2013)

Alandi, Dholka, Kandari, Kabul, Spanish Ruby, Ganesh, Bhagwa, Super Bhagwa, G137, Mridula, Arakta, Jyoti, Ruby are some of the pomegranate varieties grown in India. Ganesh and Arakta are in high demand for export (Sharma *et al*., 2014). Pomegranate farming is best suited to well-drained soils, and it prefers a hot and dry climate during fruit development. To some extent, pomegranate is considered a drought and frost tolerant crop (Kumari *et al*., 2012).

Pomegranate flowers in three seasons: January to February (Ambia bahar), June to July (Mrig bahar), and September to October (Hasta bahar). From flowering to fruit maturity, it takes approx 160-180 days. Ambia, mrig, and hasta bahar fruits are harvested between June and September, December and February, and March and April, respectively. Harvesting should be done at the optimum maturity stage since early harvesting causes improper ripening and overripe fruit harvesting causes pest and disease infestation.

Since it is a non-climacteric fruit, pomegranate fruit does not ripe after harvest. Harvesting should be done when the fruit is fully mature. Ripen fruits have a pale yellow to reddish rind and emit a metallic sound When tapped (Kumari *et al*., 2012).

Several pathogens attack pomegranates in the field and after harvest. As a result, there are significant losses during harvest and storage. One of the contributing elements to the low productivity is fruit rot. Fruit rot is very common during the rainy season and when there is a lot of moisture in the air. *Colletotrichum* spp., *Alternaria* spp., *Cercospora* spp., *Phytophthora* spp., *Penicillium* spp., *Aspergillus* spp., *Coniella granati and Rhizopus* spp. are believed to be cause of various diseases in pomegranate (Munhuweyia *et al*., 2016). *Colletotrichum gleosporioides* (Penz.) Penz. & Sacz, which causes antracnose, and *Alternaria alternata*, that causes heart rot, are two of the most common fruit rot pathogens in pomegranates. In India, *Colletotrichum gleosporioides* responsible for leaf spot disease was first time reported on coffee by Butler (1918) and anthracnose disease on mango by McRae (1934). Chandra and Tondon (1965) reported *C. gloeosporioides* causing pomegranate leaf spot and fruit rot for the first time in India. Madhukar and Reddy (1976) were the first to report *Alternaria alternata* on pomegranate leaves, and *Alternaria alternata* on fruit was first recorded in Israel, causing black spot disease (Ezra *et al*., 2010).

Heart rot can be distinguished from anthracnose based on symptoms. Deep sunken lesions on the fruit peel around the calyx area and other areas of the fruit surface, as well as necrotic patches on the leaves, are signs of anthracnose infection. Pathogens have been known to infect the mesocarp, whitish membrane, and arils in extreme cases, Pomegranate fruit rot disease, known as “heart rot” or “black heart,” is a major pomegranate disease that impacts production worldwide. Heart rot is characterized by

black rot of the fruit core that spreads from the calyx area, whereas the outer peel and the hard rind retain their healthy appearance Many fungi are responsible for pomegranate fruit rot, including *Penicillium, Aspergillus, Botrytis*, and *Rhizopus* species, however *Alternaria alternata* has been recognized as the disease's causative pathogen by many researchers. In favorable conditions, heart rot has resulted in yield losses of 15-80%. (Kumar *et al*., 2017).

As very scanty work was done on this aspect, the main theme of research is planned for identifying the causal agent of the heart rot disease and determining the morpho-cultural characteristics of pathogen along with its remedial measures with following objectives.:

1. To isolate, identify and prove pathogenicity of *Alternaria alternata* causing heart rot of pomegranate.
2. To study morpho-cultural characteristics of *Alternaria alternata.*
3. To evaluate *in-vitro* efficacy of different bio agents, essential oils and phytoextracts against *Alternaria alternata.*

CHAPTER - II

REVIEW OF LITERATURE

CHAPTER – II

REVIEW OF LITERATURE

Pomegranate is one of India's most important fruit crop. *Alternaria alternata* (Fr.) Keissler causes *Alternaria* leaf spot and fruit rot/spot, which is a severe foliar disease seen in practically every section of the world where pomegranates are grown. While examining the literature, studies on survey, disease development, pathogen characteristics, and management were taken into consideration and are provided below.

2.1 Occurrence, distributionandyieldlosses.

Madhukar and Reddy (1976)declared first time *A. alternata* as cause of leaf spot disease on pomegranate in India. However, fruit rot was not included in this report.

Shinde (1991) reported that*A. alternata*, was responsible for foliar blight of marigold (*Tagetes erecta*).

Smitha *et al*. (1998) declared*A. alternata* as disease causing fungal pathogen to chrysanthemum and zinnia.

Ghosh *et al*. (2002) reported*A. alternata*, a cause of leaf spot of gerbera grown in the greenhouse.

Mirkova and Konstatinova (2003) reported that *A. alternata*, was observed as main causal agent of leaf spot of gerbera, in Bulgaria.

Fruit rot of pomegranate caused by *Alternaria* sp. has previously been reported in USA and Mexico (Farr *et al.*, 2007) and as a postharvest disease in Greece (Pantidou, 1973).

Arunkumar (2008) declared that leaf blight of chrysanthemum incited by *Alternaria alternata* as one of the most destructive foliar disease and caused about 80-90 per cent flower yield losses in field as well as in market.

During September and October 2005 and 2006, a fruit rot due to *A. alternata* on pomegranate orchards was continuously present in the Larissa region of Central Greece,revealing yield losses of nearly 40-50per cent especially on ‘Kapmaditika’ cultivar. The internal symptoms accorded of a black rot of the fruit core started from the calyx area, while the hard, leathery rind appeared on healthy and fruits remained firm (Tziros*et al.*, 2008).

Benagi*et al.* (2009)reported 2-15 per cent disease incidence of *A.alternata* during their survey of pomegranate diseases in northern Kamatakacoverup Bagalkot, Bijapur, Belgaum, Bellary, Gadag and Koppal districts.

Ezra *et al.* (2010) declared for the first time black spot disease of pomegranate as a new disease caused by *A. alternata* in Israel. The symptoms were observed on the leaves and fruits but no damage to the inner edible tissue was observed. The causal agent was isolated and pathogenicity was confirmed.

Michailides*et al.* (2011) reported that the suspected known as black heart of pomegranate was major concern for pomegranate growers in California and proved that *Alternaria alternata* and *Alternaria* spp. were involved as main causal agent of black heart disease of pomegranate.

Khosla and Bhardwaj (2013) conducted survey of pomegranate growing areas in Himachal Pradesh to administrate the occurrence and incidence of diseases andreported incidence of leaf and fruit spots mainly by *Cercosporapunicae(Pseudocercosporapunicae)* and *Alternaria alternata* was 1.1 to 17.31 per cent during June to Sept. months.

Barbegal*et al.* (2014) decalred that *A.alternata*was responsible for causing black spot of pomegranate fruit in spain and the same pathogen causing heart rot was reported in Italy.

Kaharmanoglu*et al*. (2014) studied the heart rot of pomegranate in Cyprus (Turkey) caused by *Alternaria* spp. The incidence of heart rot was bounded in three different cultivars of pomegranate as 20.31per cent (Acco), 14.91 per cent (Herskovitz) and 9.82per cent (Wonderful).

In Spain,Vicent*et al*. (2016), discovered*Alternaria alternata*, as a cause of black heart disease in pomegranate fruit, with a disease prevalence of 10 to 15%.

Abbas *et al*. (2017) declared first time black spot of rose caused by *Alternaria alternata*,inPakistan.

Kadam *et al.* (2018) reported that among the fungal pathogens, *A. alternata* causingspotting and fruit rotting reported to be dangerous and the emerging pathogen on pomegranate.

2.2. Symptomatology.

Bedi and Singh (1972) described the symptoms of rose blight caused by *A.alternata*. As leaf margin browning from apex downwards to the base,. the lesions extend towards the centre in humid weather, leaf tip and margin became brittle and leaf colour changed from yellowish brown to dark brown.

Sahni (1973) reported the symptoms of *Alternaria* blight of rose caused by *A. alternata*. The symptoms produced as small, oval to irregular brown lesions at margin as well as at apex of the leaf, which later covered all over the leaf surface. Such spots scattered and enlarged showing concentric rings and finally the infected leaves dried and dropped down.

Srivastava and Mathur (1979) described the symptoms of jasmine leaf spot caused by *A. alternata* such marginal spots initiated from tip of the leaf or petiolar end, the lesion were brown and turned into dark brown.

Mallikarjun (1996) described the symptoms of turmeric leaf blight caused by *A.alternata* as light brown coloured spots with a clear yellow halo, circular to irregular, measuring 0.5 mm to 10 mm on leaves.

Ellis (1998) described the symptoms of chrysanthemum leaf blight caused by *A. alternata* on flowers, stems, leaves. On leaves spots were round, initially pale grey later turned grey or brownish black with whitish spot in the centre surrounded by pale and dark concentric rings.

Atia and Tohamy (2004) described the symptoms of okra leaf spot induced by *A. alternata;* as initial light brown spots on leaves which later turned to concentric dark brown spots of varying size covering large leaf areas and in severe infection, the infected leaves became brown, dried and die.

Pala *et al*. (2009) declared that *A. alternata* which induced fruit rot in pomegranate showed symptoms on leaves, flowers and young fruits. Initially uneven small dark lesions were coalesced and covered half portion of leaves and then the flowers and small fruits were found infected.

Symptoms on pomegranate as in the form of black spot induced by *A. alternata* was described as single to many black spots surrounded by green to yellow halo

on fruits and leaves. Edible part of the fruit were not damaged as the spots were only restricted to peel surface.(Ezra *et al*.,2010)

Arain *et al.* (2012) described the symptoms of okra leaf spot induced by *A.alternata* as light brown spots on leaves, later turned to dark brown spots of varying size, spread covering large leaf area and such infected leaves turned brown and died.

Nagrale*et al*. (2012) described the symptoms of gerbera leaf blight incited by *A. alternata* as light to dark brown, roundish-oval to irregular spots on leaves which coalesced and produced shot hole symptoms during severe infection.

Farwood and Hadian (2012) described the symptoms of gerbera leaf blight induced by *A. alternata* as initially small, brown, scattered round to irregular spots on the leaves which coalesced covering leaf area followed by defoliation.

Gat *et al*. (2012) mentioned that symptoms of *A. alternata* were appeared as uneven lesions surrounded by yellow halo, round lesions on fruits where damage was restricted to peel. Symptoms of black spot and fruit rot of pomegranate were restricted to internal area where as peel and leaves were uninfected.

Khosla and Bhardwaj (2013) declared that symptoms of *Alernaria*leaf spotof pomegranate in which leaf spot includes small, light brown round or irregular spots having concentric rings in center which coalesce and form big spot of dark browncolour. Affected leaves became yellow and drop down. Small brownish spot of irregular shape were developed on fruits which becames large with black margins and rotting in interior fruit portion.

Ammar and El-Naggar (2014) reported the appearance of novel symptoms of *Alternaria* spp. on pomegranate fruit. These symptoms include black spots on fruit, ranging from a single lesion to lesions that cover more than 50% of the fruit surface. The damage was restricted to the peel surface while the edible tissue remains unaffected. In contrast, heart rot of pomegranate associated with *Alternaria* spp., and *Penicillium funicufosum*, in which the fruit rot, was restricted to the internal area whereas the peel remain unaffected.

Kantwa*et al*. (2014) described the symptoms of groundnut leaf blight induced by*A. alternata*. On foliage small, oval , discoloured, scattered irregularly lesions

appeared enlarged, became irregular, turned brown to grey with yellow zone surrounding the spots.

Ezra *et al.* (2015) declared that one side of heart rot infected pomegranate fruits exhibited redness. Such fruits emit hollow sound when tapped.

Faedda*et al.* (2015) described the symptoms of heart rot and dry black rot of aril tissues originating from calyx area confined to compartments of pomegranate and rind remains unaffected. Mature fruits infected with *A. alternata* were lighter in weight.

Gaikwad and Karande (2016) described the symptoms of pomegranate fruit spot caused by*Alternaria alternata.*Small reddish brown circular spots were appeared on the fruits. As the disease advanced these spots, coalesced to form larger patches and the fruits started rotting. The arils get affected which became pale and became unfit for consumption.

Priyanka *et al.* (2018) described the symptoms of leaf spot of marigold incited by *A. alternata* as initially necrotic spot appeared which later it progresses as leaf blight and completely coalesced leading to drying of entire leaf.

Kahramanoglu*et al.* (2018) reported that heart rot or black heart damage in pomegranate started as aril decay with no external symptoms. But infected fruits showed unusual skin colour and abnormal shapes which leads to reduced marketable quality.

2.3.Isolation and Identification of Pathogen.

Solanki (2004) isolated *A.alternata*, responsible for leaf spot of gerbera. The fungus produced abundant mycelial growth on PDA media which gradually turned light grayish and brown to dark grayish in colour within ten days.

Ezra *et al*. (2010) isolated *Alternaria*spp from infected pomegranate fruits and identified as *Alternaria alternata* based on fungal colonies emerged from symptomatic tissue had morphology and conidia typical to *Alternaria* spp. Sequence analysis of rDNA ITS region and β-tubulin gene of four different isolates exhibited 100 per cent identity to *Alternaria alternata*.

Farhood and Hadian (2012) isolated *A. alternata*, which caused leaf spot of gerbera. The fungus produced abundant, olivaceous black colonies with dark olivegreen margins, and abundant branched septate mycelium. The conidiophores were pale brown to olive brown, branched and straight.

Gat *et al.* (2012) used infected fruits, leaves and branches of pomegranate orchard for the isolation of *A. alternata* on PDA media by tissue isolation method. Single spore isolation technique was used to get pure culture.

Ezra *et al*. (2015) isolated fungus from both soft and dry rotted arils and identified as species of *Alternaria*. Several single spore *alternaria* culture was examined through sequencing of ITS 5.8S rDNA. All the isolates used in this study exhibited 99 per cent identity to sequences of *A. alternata*.

Faedda*et al.* (2015) isolated *A. alternata* from heart rot infected pomegranate fruits. Fungal culture was obtained on potato dextrose and potato carrot agar by following tissue isolation technique. Hypha or conidium transfer methods were followed to get an auxenic culture.

Sarkar *et al*. (2017) isolated *A.alternata* inducing leaf spot of chilli. The fungus produce initially white colony, cottony with abundant aerial mycelium which gradually turned grey. Conidiophores were short to long, simple or branched. Conidiophores were golden to brown coloured with 2-9 transverse and 0-2 longitudinal septa. Conidia were born in long chains on conidiophores, which were brown, thick walled having beak.

Kumar *et al*. (2017) collected symptomatic fruits from pomegranate orchard and isolated *A. alternata* on PDA and incubated at 25ºC. Hyphal tip isolation method was followed to obtain pure culture.

Zade *et al*. (2018) isolated *A. alternata* from infected soybean leaves. Based on colony and morphological characters of the fungi it was identified as *Alternaria alternata*.

Priyanka *et al*. (2018) isolated *A. alternata*, from infected leaves of marigold. The fungus produced greyish black to black coloured colonies. Mycelium was septate, branched and gray-brown to olivaceous with or without zonation.

Nivedha*et al*. (2019) isolated *A. alternata*, causing leaf blight of jasmine and reported that the fungus produced abundant brownish septate mycelium and conidia were olive- brown, produced in chains having both vertical and horizontal septations.

Rasheed *et al*. (2019) isolated *A. alternata*, causing Aloe vera leaf spot. The fungus developed profuse growth of mycelium on PDA medium,where mycelium was hyaline that changed to grey- brownish, septate, multi-celled, and branched.

2.4. Pathogenicity:

Bedi and Singh (1972) confirmed the pathogenicity of *Alternaria alternata* on rose by inoculating spore suspension of the pathogen and the typical symptoms were started after 6 days of inoculation.

Sreekantiah *et al.* (1973) justified the pathogenecity of *Alternaria alternata* by inoculating spore suspension on leaves and fruits of chilli seedlings.

In Italy, Wada *et al*. (1996) confirmed the pathogenicity of *A. alternata* by spray inoculation method following Koch's postulate in strawberry.

Khodke *et al.* (2000) isolated *A. alternata*, causing leaf spot of chilli and confirmed its pathogenicity. Circular brown or black spot of varying size with concentric zonation on chilli leaves were observed.

Akhtar *et al.* (2004) confirmed the pathogenicity of *A. alternata* causing leaf blight of tomato by spray inoculating spore suspension.

Chitra *et al*. (2006) confirmed the pathogenicity of *A. alternata* causing leaf blight of groundnut, by spray inoculation of spore suspension.

Singh *et al.* (2006) justified the pathogenicity of *A.alternata* isolates on leaves and inflorescence of Adhatoda.

Mangala *et al*. (2006) isolated and confirmed the pathogenicity of *A. alternata* causing leaf blight of chilli. Typical leaf blight symptoms were developed within fifteen days after inoculation.

Tziros *et al.* (2008) confirmed the pathogenicity of *A. alternata* causing fruit rot of pomegranate and identified the pathogen on the basis of their morphological characteristics.

Ezra *et al.* (2010) conducted an experiment to justify the pathogen causing black spot of pomegranate as *A. alternata* by detached fruit and leaf method.

Gat *et al*. (2012) confirmed the causal agent for the black spot of pomegranate as *A. alternata* on detached leaves, fruits and flower by artificial inoculation of fungal spore suspension.Pomegranate seedlings were also used to prove the

pathogenicity of *A. alternata* causing pomegranate heart rot by following detached fruit technique. (Ezra et al.,2015)

Ramjegathesh and Ebenzer (2012) confirmed the pathogenicity of *A. alternata*inducing leaf blight disease of onion by spray inoculation of conidial suspension.

Nagrale *et al.* (2012) confirmed the pathogenicity of *A. alternata* inducing blight of gerbera by spraying spore suspension. Typical blight symptoms on leaves as well as flower stalks were developed within 8-10 days of inoculation.

Kumar *et al*. (2012) justified the pathogenicity of *A.alternata* inducing leaf blight of groundnut by inoculating conidial suspension. The typical symptoms were appeared within 12-15 days after inoculation.

Nagrale *et al.* (2013) confirmed the pathogenicity of *A.alternata* causing blight of Gerbera by microdroplet inoculation technique and by mycelial bit inoculation method.

Khodaei and Arzanlou (2013) proved pathogenicity on sunflower by spraying with *A. alternata* at 10^5 conidia/ml suspension. After two days of inoculation disease symptoms were started as small chlorotic spots distributed evenly on blade of lower leaves. The spots gradually changed to irregular, necrotic, pale to dark brown and darkening outwards spots, which were surrounded by a yellow halo.

Kumar *et al.* (2017) collected fresh, healthy and fully grown pomegranate fruits and confirmed the pathogen associated with black spot as *A. alternata*. These collected fruits were injured artificially and inoculated with isolated pathogen and incubated for 5-6 days. The fungus was re isolated and observed for morphological characters.

Priyanka *et al.* (2018) proved the pathogenecity of *Alternaria* spp. causing leaf and flower blight of marigold by inoculating spore suspension.

Nivedha *et al*. (2019) confirmed the pathogenicity of *A. alternata* causing, leaf blight of jasmine, by spraying conidial suspension.

2.5.Morpho-cultural characteristics of *Alternaria* spp.

Keissler (1912) described morphology of *A. alternata* as black or olivaceous black and sometimes grey colonies. Conidiophores were produced singly, simple or branched, straight or flexuous, sometimes geniculate, pale to mid olivaceous or brown, smooth, measuring 50 µm long, 3-6 µm thick. Conidia formed in long, branched chains, obclavate, pyriform, ovoid or ellipsoidal often with short conical or cylindrical beak, not more than one third length of the conidium, pale to mild golden brown, smooth or curved with eight transverse and usually several longitudinal or oblique septa.

Chowdhary and Varshney (2000) and Zang (2003) reported that, morphological characters of different structures such as mycelium as young and matured, conidiophores, conidia were noted by adopting slide culture technique in *Alternaria alternata* (Fr.) Keissler causing blight of gerbera.

Mirkova and Konstantinova (2003) stated the morphology of *A. alternata* from gerbera. It was reported that conidia were in chains, sometimes branched chains of 5-12 spores. The conidia were variable in size and shape and usually ovoid to ellipsoid or obclavate and with a short beak at the end. The long oval conidia were usually pale brown to brown with 3-6 horizontal and 0-2 longitudinal or slanting septa and measured 25-35 x 5-10 µm.

Solanki (2004) described the morphology of *A. alternata* causing gerbera leaf spot as septate hyphae and light to dark brown coloured. The mycelium irregularly branched at acute angle also conidiophores light brown, long conidia with 3-5 horizontal and 0-3 longitudinal septa measuring about 23.40 -32.76 X 5.27 -9.95µm.

Gohel (2004) described the morphology of *A. alternata* causing leaf spot and fruit rot of chilli. It was reported that conidiophores were brown to dark brown, upstanding, grouped, unbranched and septate i.e. 1 to 6 septa and muriform. Conidia were deep brown with 1-4 horizontal and 0-2 longitudinal septa with variable size and shape . The beak length measured as 10.83- 43.57 µm x 8.62-16.25 µm in size with average size of 28.90-12.90 µm.

Mangala *et al.* (2006) stated the morphological characters of *A.alternata* causing leaf blight of chilli. It was reported that fungal colonies were dull white coloured initially then turned olive green upon sporulation from fifth day. The conidia

wereobserved as obclavate with beak, thorny and pigmented with relatively 1-9 horizontal septa and 0-4 vertical septa and measured about 45.77- 47.85 µm in breadth.

Raja and Reddy (2007) described the morphology of *A. alternata* causing leaf spot and fruit rot of egg plant. It was reported that conidiophores were short, arised singly and measured 125.6-152.6 µm long and 5.2-7.8 µm thick. The size of conidia was varied from 35.2 to 43.5 µm long and 12.4 to13.9 µm wide. Transverse and vertical septations of conidia were ranged from 1 to 8 and 0 to 3, respectively.

Panchal (2009) reported the morphological characters of *A. alternata* causing fruit rot of tomato. It was reported that conidiophores were brown to dark brown, simple and septate. Conidia were light to dark brown, unattended or sometimes in short chain arranged in acropetal manner. Conidia wereobelavate in shape but few were oviform to pyriform in shape, broadly rounded base with 3-8 horizontal septa and several longitudinal septa with different size and shape. The sizes of the conidia measured about 13.6 to 46.30 µm x 4.6 to 14.12 µm (average 32.45 µm x 11.36 µm).

Suryawanshi*et al.* (2010) reported that *A.alternata*inducing heart rot of pomegranate showed abundant growth and sporulation on Sabouraud's agar.

Ramjegathesh and Ebenezer (2012) studied ten isolates of leaf blight of onion caused by *A. alternata*. It was reported that all the isolates produced light brown conidia with muriform shape and varied in size that are length, width and beak length of conidia.

Archana (2012) reported that among different media tested, the mean mycelial growth of *Alternaria alternata* was maximum in PDA and Sabourad's agar (90.00 mm) followed by Richards agar (82.30mm) whereas sporulation was maximum in PDA and host leaf extract+1% sucrose agar.

Nagrale*et al.* (2013) described the cultural and morphological characters of *Alternaria alternata* causing leaf blight of gerbera. It was reported that fungus produced abundant mycelium on Potato Dextrose Agar (PDA) medium with an average width of 4.42 µm in diameter. The conidiophores, conidia and intercalary chlamydospores were measured as 42.26 x 4.29 µm, 47.16 x 13.49 µm and 7.22 µm in diameter, respectively.

Shamala and Janardhana (2015) described the morphology of *A. alternata* causing leaf blight of chrysanthemum and reported that the fungus produced abundant mycelial growth on potato dextrose agar medium. Initially, the mycelium was hyaline which was later turned to grey brownish with multicelled, septate conidia. Also conidia born in chains on conidiophores, 10 or more, light olivaceous to dark brown, obclavate to ellipsoid, apex tapered, 1-3 horizontal and 2-10 vertical septa. Conidia measured about 32.42µm (12.36 – 61.76 µm) x 13.79 µm (9.1–21.1µm).

Rasheed *et al.* (2019) studied the morphology of *A.alternata* causing leaf spot of Aloe-vera and reported that the fungus produced erect, branched, golden brown coloured conidiophores, measuring 15 mm long and 2-6 mm width. The conidia were gold brownish in colour, formed in lengthy branched chains, obclavate in form with small pointed flask.

2.5.1 Molecular characterization of *Alternaria* spp.

Chavan (2004) studied the morphological, pathological and molecular characterization of *Alternaria* species causing leaf spot and blight disease of sunflower. In this studies, RAPD fingerprint analysis of 24 isolates of *Alternaria* spp. isolated from sunflower was done and based on fingerprint data, 24 *Alternaria* sp. were categorized into 07 groups. The members of these groups showed 40 to 100 per cent genetic similarity to each other.

Chavan *et al.* (2012) studied molecular characterization of six fungal pathogens *viz. Alternaria alternata* (MTCC 7202), *A. longipes*(MTCC 7150), *A. dianthicofa*(MTCC 7203), *A. longipes, Dreschsleraaustraliensis*(MTCC 7215) and *Phomaheliamhi*associated with leaf spot and blight diseases of sunflower. This was done using RAPD and RFLP analysis. Of the 18 arbitrary primers tried, OP A 11 , OP A 12, OP A 15 and OP A 19 produced significant polymorphism. The dendrogram generated based on RAPD and RFLP analysis grouped leaf spot and blight pathogens into five different clusters. Cluster I comprised of *A. longipes*and *A. dianthicola*which showed similarity of 73 to 100 per cent, whereas, *A. longipes*and *A. alternata* were found very close to member species of cluster I, while, *D. australiensis*and *P. helianthi*exhibited wide variability. Both RAPD and RFLP analysis revealed the genetic distances among species in the range 27-82 per cent and 0-74 per cent, respectively. RFLP marker based

on rDNA probe was found more informative than RAPD. Thus RAPD and RFLP could effectively discriminate all the six leaf spot and blight pathogens of sunflower at molecular level.

Kakvan*et al.,* (2012) analyzed genetic diversity among different pathotypes of *Alternaria alternata,* causing various diseases in citrus including *Alternaria* brown spot of tangerines, leaf spot in rough lemon and *Alternaria* black rot on citrus fruits by using RAPD-PCR. In this studies, 15 random tenmer primers were used and further cluster analysis was performed using NTSYSpc V2.2. Based on pathogenic potential of *Alternaria* isolates, all isolates were categorized into three distinct group *viz.,* severe, moderate and low pathogenic. Similarly based on dendrogram analysis it was revealed that from the cluster analysis of isolates, these were classified into five groups which showed 85 per cent similarity level. Molecular diversity of isolates was found highly related to geographical region, host and partly virulence of isolates.

Nasim *et al.* (2012) studied molecular polymorphism and phylogenetic relationship amongst 10 isolates of *Alternaria alternata* collected from Pakistan. Based on data generated by primer GL-A-0 I, among ten isolates, five were able to generate 22 amplicons of size in the range of 250-1200 bp. Among 22 amplicons, 19 were found itrogenic, while 3 amplicons (250 bp, 600 bp and 1200 bp) were polymorphic. Furthermore, the cluster analyses based on similarity coefficient, the isolates were categorized into two distinct groups.

Abeer*et al.* (2014) extracted DNA samples from mangrove leaves, healthy as well as artificially infected with *A. alternata*. Seven forward and reverse primers were used in this study. PCR amplification with primer Al-f1/Al-r1 expressed 370 bp amplification product in both *A. alternata* isolates and the artificially infected mangrove leaves only, whereas, no amplified product was detected in case of healthy mangrove leaves with the diagnostic primer pair (Al-f1/Al-r1).

Aloi*et al.* (2021) extracted DNA from pomegranate fruit infected with *Alternaria* spp. using DNA isolation Kit. A multilocus approach was adopted to characterize and determine the phylogenetic allocation of the 42 isolates from pomegranate fruit. Portions of four *Alternaria* barcoding genes/regions, i.e. internal transcribed spacer (ITS), translation elongation factor 1-α (EF-1α), glyceraldehyde-3-

phosphate dehydrogenase (GAPDH) and one SCAR marker (OPA10–2), were sequenced. The primers used for amplifying these genes/regions were ITS1/ITS4 for ITS, EF1-728F/EF1-986R for EF-1α , GPD1/GPD2 for GAPDH and OPA10-2R/OPA10 s. According to this analysis, 38 out of the 42 isolates were associated with *A. alternata.*

2.6.*In-vitro*bioefficacy of bioagents

Ghosh *et al.* (2002) assessedvarious fungal bioagents *in vitro* against *A. alternata* causing leaf spot of Gerbera and reported significantly highest mycelial growth inhibition with *T. viride(*87.49%), followed by *A. awamori* (85.70%), *T. hamatum* (83.32%) and *T. harzianum*(50.00%).

Gohel*et al*. (2005) studied efficacy ofvarious fungal antagonists *in vitro* against *A. alternata*, causing leaf spot and fruit rot of chilli and reported that significantly highest mycelial growth inhibition was observed in treatment*A. flavus* (77.00%) followed by *T. longibrcchiatum*(73.00%) and *T. harzianum*(49.00%).

Ahire*et al.* (2012) assessed various fungal bioagents *in vitro* against *A. alternata* causing blight of marigold and reported that significantly highest mycelial growth inhibition was occurred by *T. koningii*(80.55%), followed by *T. hamatum* (67.03%) and *G. virens* (22.77%).

Mumtaz *et al*. (2012) evaluated various fungal bioagents *in vitro* against *A. alternata* in oilseed crops and reported that significantly highest mycelial growth inhibition was recorded with *Trichoderma viride*(67.6%), followed by *Pseudomonas fluorescens* (66.00 %) and *Bacillus subtilis* (53.3%).

Pareek *et al*. (2012) evaluated various fungal bioagents *in vitro* against *A. alternata* causing leaf spot of cucumber and reported that*Trichoderma harzianum*was most effective over other treatments and produced inhibition zone of (74.00 mm) followed by *T. viride*(63.00 mm) and*Aspergillus niger*(37.5 mm).

Baig *et al*. (2012) evaluated *Trichoderma viride, Trichoderma harzianum, Pseudomonas fluorescens* and *Bacillus subtilis* against *Alternaria alternata* in oilseed crops and the results revealed that *Trichoderma viride*was most effective with significant mycelial inhibition of 67.6 per cent followed by *Pseudomonas fluorescens* (66%) and *Bacillus subtilis* (53.3%).

Apet *et al.* (2014) evaluated various fungal bioagents *in vitro* against *A. alternata*, causing leaf spot of gerbera and reported that highest mycelial growth inhibition was observed with *T. viride*(86.67%), followed by *T. hamatum* (78.34%), *T. koningii*(76.67%), *T. lignorun*(68.15%), *T. harzianum*(53.16%) and *Pseudomonas fluorescens* (50.38%).

Hansraj and Sharma (2017) studied the efficacy of different bio agent against *Alternaria alternata* causing core rot of apple and reported that among different bio agents *Trichoderma hamatum* exhibited maximum mycelial growth inhibition (55.13%) in vitro.

Zade *et al*. (2018) evaluated various fungal bioagents *in vitro* against *A. alternata* causing leaf spot of soybean and reported that significantly highest mycelial growth inhibition was recorded with *Trichoderma harzianum*(79.65%) followed by *Trichoderma asperellum*(76.55%), *Pseudomonas fluorescens*(68.68%) and*Trichoderma viride*(64.65%).

Kadam *et al.* (2018) tested the efficacy of bio-agents and botanicals *in vitro* against *A.alternata* causing leaf spot of pomegranate. Among the tested bio agents*T. viride* recorded significantly highest mycelial growth inhibition (86.85 %) followed by *T. hamatum*(82.04 %) and *A. niger*(81.11 %).

Marchande *et al*. (2020) evaluated bacterialandfungal bioagents *in vitro* against *Alternaria alternata.* The results revealed that *Trichoderma virens* was found most effective with significantly least mycelial growth i.e. 33.4 mm and highest inhibition of mycelial growth i.e.(62.88%), followed by *T. harzianum*(62.33%), *A. niger*(38.52%),*P. fluorescens* (25.17%) and *B. subtilis* (20.67%).

2.7 *In-vitro* efficacy of plant extracts.

Karade and Sawant (1999) testeddifferent plant extracts on solid and liquid media against *A. alternata* and reported that *Alliumsativum* was found highly effective against the pathogen.

Pareek *et al.* (2012) evaluated five different plant extracts against *A.alternata*of cucumber at 5, 10 and 15 percent concentrations. Results revealed that

Allium sativum recorded 71.23 per cent and *Azadirecta indica*57.10 per cent inhibition of mycelia growth.

Amrate *et.al.* (2013) reported that among all tested plant extracts turmeric rhizome and garlic clove extract were found to inhibit about 70.00 percent growth of pathogen at 20 percent concentration. Garlic oils at 1000 Ug/mL, whereas lemmon grass, ginger, palmarosa and tulsi oils at 2000ug/mLhad completely restricted the growth of pathogen.

Bhosale *et al.* (2014) tested different aqueous leaf extract against *Alternaria alternata* of soybean. It was reported that neem, ginger and eucalyptus were found highly inhibitory to mycelial growth of *A.alternata.*

Kantwa*et al*. (2014) conducted an *in vitro* study to determine the efficiency of several plant extracts against *Altetrnaria alternata* causing groundnut leaf blight. The results revealed that among the plant extracts tested garlic clove extract was inhibited mycelial growth by 46.60 per cent of *Alternaria alternata* followed by neem (43.30%) and datura (40.30%).

Regmi*et al.* (2014) reported that leaf extract of *Jatropacurcas* showed maximum mycelial growth inhibition of *Alternaria alternata* (62.9%) followed by *Datura strumarium* leaf extract (55.6%) and *Azadirachata indica* (51.9%).

Singh *et al.* (2014) evaluated six methanol basedplant extracts against *Alternaria alternata* at concentrations of 5, 10, and 20 per cent. The results revealed that oleander showed greatest reduction in mycelial growth, followed by Parthenium, Ocimum, Lantana, Vernonia, and Eucalyptus.

Rai *et al*.(2017) evaluated three botanicals(Neem, Garlic and Onion)against *Alternaria* spp.inducing*Alternaria* leaf spot in cabbage. Results showed that Neem leaves extract at 5 per cent inhibited the development of *Alternaria* spp by 66.83 per cent followed by Onion bulbs extract at 4 per cent (38.12%) and Garlic bulb extract at 3 per cent (25.95%).

Jakatimath*et al*. (2017) evaluated different botanicals *in-vitro* for control of *Alternaria alternata* and found that onion and garlic bulb extract inhibited maximum mycelial growth of *Alternaria alternata* both at 5 and 10 per cent concentration.

Rajhans and Sharma (2017) evaluated seven different plant extract at different concentration against *Alternaria alternata*causing core rot of Apple. Out of which *Azadirachta indica* recorded 84.80 per cent mean mycelial growth inhibition of pathogen.

Nidhika Rani *et al*. (2018) reported that garlic clove extract at 10per cent concentrations exhibited the maximum inhibition (84.31%) against *Alternaria alternata,*whereas 82.18 per cent inhibition of mycelial growth was observed in case of *Alternaria tenuissim*. The onion and neem extract were also found better treatment in inhibition of both pathogens.

Zade *et al.* (2018) evaluated eleven plant extract *viz*., Neem, Congress grass, Ghaneri, Mehandi, Dhatura, Garlic, Nilgiri, Beshram, Turmeric, Ginger, against *Alternaria alternata* isolated from soybean leaves by poisoned food technique. The results revealed that among the tested plant extracts, garlic clove extracts at 10 per cent recorded maximum inhibition (87.50%) of mycelial growth of test fungus and was significantly superior to rest of the treatments. This was followed by Neem leaf extract and Onion bulb extract which recorded 47.34 and 43.47 percent mycelial inhibition of *A. alternata* respectively. Rest of the plant extracts recorded percent inhibition in the range of 11.23 to 39.89 per cent against test pathogen.

Kadam *et al*. (2018) evaluated eleven different plant extreact at 10 per cent concentration against *Alternaria alternata*. Out of which *Allium sativum* recorded highest mycelial growth inhibition i.e. 72.41per cent, whereas as *Azadirecta indica* recorded 66.67%, *Eucalyptus globules*60.37 per cent and *Curcuma longa* recorded 52.96 per cent mycelial growth inhibition.

2.8 *In-vitro* efficacy of essential oils.

Feng *et al*. (2011) evaluated the efficacy of thyme oil*in vitro* and *in vivo*against *Alternaria alternata* (Fr.) Keissl. The results showed that Thyme oil possessed great fumigant and contact toxicity against *A. alternata* at different concentrations *in vitro*. The growth was fully suppressed 6 hrs after inoculation in potato dextrose broth at 2000µL/L.

Barman *et al.* (2015) tested seven plant oils against *A. alternata* inducing early blight of tomato.Results showed that eucalyptus oil and neem oil @ 0.05 and 0.1

percent inhibited mycelialgrowth by 97.2per cent and 95.1 per cent respectively, followed by karanj oil(84.40%), lemongrass oil(76.40%), zinger oil(69.30%), patchouli oil(62.70%), and garlic oil(55.30%).

Soyluand Kose(2015) investigated essential oils *in vitro* conditions against*Alternaria alternata* inducing post-harvest fungal disease of Citrus. The results indicated that *Origanumonites* and *Tymbra spicata* exhibited the strongest antifungal activity against *A. alternata* by inhibiting complete inhibition of mycelial growth of *A.alternata* at 20.0 and 80.0 µg /ml concentrations, respectively.

Gadhi*et al.* (2020)tested some essential oils such as Castor, Jasmine, Clove, Sesame, Neem, Coconut, Henna, Black seed, and Mint oil at different doses of 1, 2, 4, and 6 per cent against *A. alternata*. The results showed that maximum colony growth inhibition of *A. alternata* was recorded by Sesame(80.00%), followed by Coconut (77.04%), Henna (72.59%), Mint (66.07%), Black seed (71.85%), Jasmine (64.07%), Clove (70.74%), Neem (73.33%) and Castor (58.89%), whereas minimum inhibition (1.00%) was recorded in control.

Awais*et al*. (2020) showed that plant essential oil of Karanj at all concentrations significantly inhibited the mycelial growth (89.4, 92 and 96.2 percent) in contact assay method as well as 96.2, 97 and 98.2 % growth inhibition regarding fungal culture transfer(FCT) experiment while, in case of well diffusion method (32%), (41%) and (48%) growth inhibition was recorded at 7 th day of incubation followed by Moringa essential oil and Kikaressential oil as compared to control that showed 0 % growth inhibition. Moreover, results related to spore germination assay revealed that Sukh chain essential oil at 200, 400 and 600 ppm showed significant inhibition of germ tube length of *A. alternata* (140.8 µm, 77.5 µm and 34.1 µm) respectively as compared to control in which germ tube length was recorded 250 µm. It was concluded that Karanjessential oil has a great potential to inhibit the growth of *A. alternata.*

Nizamani*et al*. (2020) evaluated different essential oils againt*Alternaria alternata* causing fruit rot of Jujube. The results showed that minimum colony growth of A*lternaria alternata* (31.60, 21.25 and 15.16 %) examined in clove oil at 5%, 10% and 15% concentration respectively followed by Neem oil (42.60, 31.60 and 21.30%)

respectively and maximum colony growth of *A. alternata* (62.71, 52.40 and 41.75%) was observed under castor root oil at 5%, 10% and 15% concentration respectively.

CHAPTER - III

MATERIAL AND METHODS

CHAPTER – III

MATERIAL AND METHODS

The experimental studies were conducted on heart rot of pomegranate during the year 2020-2021. Isolation, identification, pathogenicity test, physiological and cultural studies, *in vitro* evaluation of phytoextracts, essential oils and bio agents against the pathogen were carried out at Department of plant pathology, College of Agriculture, VNMKV, Parbhani and National Research Centre on Pomegranate, Solapur. The material used and methods followed for these studies are given herein.

3.1 General laboratory procedures

3.1.1 Glassware cleaning.

Corning and Borosil glassware were utilized in all of the laboratory investigations. The glassware was immersed overnight in a cleaning solution made up of 60 g potassium dichromate ($K_2Cr_2O_7$) and 60 ml concentrated sulphuric acid (H_2SO_4) dissolved in one litre of distilled water. They were then washed with detergent, rinsed many times in running tap water, then rinsed again in distilled water as needed for various studies.

3.1.2 Sterilization.

All of the glassware used in the research were sterilized in hot air oven at 180°C for 2 hrs. Both solid and liquid media were sterilized in autoclave under 15 lbs. of pressure for 20 minutes.

3.2 Isolation of pathogen and pathogenicity.

The pathogen that causes heart rot was isolated from an infected leaf and fruit samples using a conventional tissue isolation procedure. For pathogen isolation, the pathogen was isolated from pomegranate leaves and fruit that showed typical indications of heart rot/black heart.

Using a sterilized razor blade, the leaf and fruit samples showing symptoms of heart rot were sliced into small pieces of 2.5-5.0 mm in size, with some healthy and some contaminated portions. The isolation method was completed in an aseptic

environment of laminar air flow cabinet. Surface sterilization of the laminar air flow bench, needle and hands was done with 70 per cent ethanol or spirit. The small pieces of fruits were disinfected for 1 minute with a 0.1 per cent solution of Sodium hypochloride (NaOCl), then rinsed three times in sterile distilled water to eliminate any remaining traces of mercuric chloride. To remove the water, the fragments were dried on sterile blotting paper. These bits were placed in sterile Petri dishes containing 15-20ml solidified Potato Dextrose Agar (PDA) medium and incubated at 28±2°C in BOD incubator for 6-7 days to see the growth of pathogen. When the mycelia began to grow around the host tissue, it was transferred to PDA slants for further research.

3.2.1 Single spore Isolation

10 mL of 2 per cent sterilized water agar was placed into Petri plates and allowed to solidify. Using sterile distilled water from a 12 days old culture, a dilute spore suspension was made. One ml of suspension was evenly distributed in Petri plates and 2 per cent water agar was poured aseptically over it and allowed to solidify. The plates were next inspected under a compound microscope with a low power objective (10x) to locate the conidia. A single isolated conidium was then marked with ink on the surface of plate under the microscope. These designated agar patches were cut and transferred to PDA slants under aseptic circumstances using a cork borer (2 mm) and incubated in BOD incubator at 28±1°C. Pure cultures derived from such slants were used for further studies.

3.2.2 Identification of the pathogen.

The study was carried out in order to confirm the identification of pathogen. The fungus was identified after studying one hundred conidia from a mature pure culture of the fungus collected from infected pomegranate leaves and fruits under a microscope with a low power objective (10x). The fungal dimension was measured using a stage and an ocular micrometer in terms of length, breadth, beak length, and the number of septa on the fungus. The conidial average length and breadth, as well as the number of septa, were measured. To identify the pathogen, the findings were compared to standard measurements given by Ellis (1971). The pathogenic identity of *A. alternata* thus confirmed was used for further studies.

3.2.3 Maintenance of the culture

The pathogen was sub cultured on PDA slants and allowed to grow at 28°±2°C and renewed once after every 30 days. After three months, the virulence of fungus was maintained by inoculating the host with pathogen.

3.2.4 Proving the pathogenicity

Healthy seedlings sprang out of air layers of a pomegranate that was 4-5 years old were raised in a 6" x 5" polyethene bag filled with sterile soil. Plants were thoroughly cleaned with sterilized distilled water and wiped with a moist cotton swab. For 24 hours they were wrapped in plastic bags to create humid condition. The plants were sprayed with an inoculum suspension made from a 12 days old culture in sterile distilled water. To ensure that the pathogen penetrated the tissue successfully, the seedlings were wrapped with polythene bags and incubated for 48 hours. For comparison, control plants that had not been inoculated with the test organism were sprayed with sterile distilled water. After five days, the seedlings were taken from the polythene bags and stored in a greenhouse (30±1°C and 80% RH). The emergence and progression of symptoms were monitored on a regular basis.

Pin prick and cotton swab tests were used to demonstrate pathogenicity. Uninjured, ripe and disease-free fruits were collected from the field and properly washed with tap water before being swabbed with 1 per cent mercuric chloride (NaOCl) in the laboratory. The fruits were rinsed in sterile distilled water to eliminate any excess traces of mercuric chloride. Inoculation was done with sharp sterilized tooth pricks. A suspension of spores from a fungal culture was created. By swabbing cotton over perforated pomegranate fruits, healthy pomegranate fruits were infected with this spore suspension. As a control, sterile water was sprayed over the fruits.

The appearance of symptoms on inoculated leaves and fruits was seen seven days following inoculation in both cases. The pathogen was re-isolated from these artificially inoculated leaves and fruits using the conventional tissue isolation procedure. After that, the acquired fungal culture was sub cultured on PDA medium. The original isolated pathogen was compared to the re-isolated pathogen. These cultures were then employed in subsequent research and were kept in slants at 4°C.

3.3 Morpho-cultural variability among *A. alternata* isolates

3.3.1Cultural variability

Eight isolates of *A. alternata* (Alt-1 to Alt-8) were cultured on autoclaved and cooled PDA in sterilized glass Petri plates (90 mm diam.) collected from eight different locations. These PDA plates were inoculated using a mycelial disc (5 mm) derived from an actively growing week-old pure culture of the test isolates separately and incubated at 27 $\pm$ 1 °C

After a week of incubation, observations on culture / colony characteristics such as colour, look, shape, zonation and margin of the colony, and growth rate / 24 hr. were recorded. After 12 days of incubation, sporulation was seen. At 12 days of incubation, the sporulating culture of the test isolates in Petri plates was flooded with 10 ml distilled water and gently scraped with a camel hair brush to extract spore suspension for counting sporulation. A temporary mount of the spore suspension on a glass slide was created, placed under research microscope, and the spores were counted in five random microscopic fields (400x) and averaged, and the sporulation frequency was classed as per the grades suggested by Kumar and Choudhary (2006).

Grade	Sporulation	No. of spores/ microscopic field
-	Absent	Nil
+	Poor	1-10
++	Fair	11-30
+++	Good	31-50
++++	Excellent	More than 50

3.3.2 Molecular identification of the pathogen.

Extraction of fungal DNA by modified CTAB method.

The DNA of *Alternaria alternata* was isolated. The isolation of DNA from filamentous fungus is difficult because of high polysaccharide content in rigid cell wall. Therefore, for fungal DNA isolation the modified CTAB technique was used.

Buffer preparation

For 250 ml 2X CTAB

- 2%CTAB- 5gram
- 100mM tris (pH 8)-25 ml (0.5 M)
- 20mM EDTA-10 ml (10M)
- 1.4 M Nacl-35 ml (10M)

10% CTAB -100 ml

- CTAB-10 gram
- 0.7 M Nacl-7ml (10M)

Procedure:

In a mortar and pestle, one hundred mg of fungal tissue was crushed to a fine powder using liquid nitrogen, then transferred to a 2 ml microcentrifuge tube containing 750 µl extraction buffer and 5 µl RNase and combined by inversion. This mixture was incubated for 30 minutes at 65°C in hot water bath and then centrifuged for 10 min. at 10000 rpm. The upper aqueous phase was then collected in a new microcentrifuge tube and an equal amount of Chloroform: Isoamyl alcohol was added to the aqueous phase, which was centrifuged at 13000 rpm for 15 minutes. The upper aqueous phase was collected into a fresh tube, which was then filled with an equal amount of the Chloroform: Isoamyl alcohol mixture and mixed by inversion. For 15 minutes, the mixture was centrifuged at 13000 rpm. The top aqueous phase was then collected into a new 1.5 ml microcentrifuge tube, which was then filled with an equivalent amount of chilled Isopropanol and maintained at -20°C for 2 hours to precipitate the DNA. After centrifuging the tubes at 14000 rpm for 10 minutes, DNA pellets were obtained. It was then rinsed in a 70 per cent ethanol solution. Air dried DNA pellets were dissolved in 30 µl 1X TE buffer.

3.3.3 Polymerase chain reaction:

The polymerase chain reaction (PCR) is a molecular biology technique for amplifying a single or few copies of DNA over several orders of magnitude, resulting in thousands to millions of copies of a specific DNA sequence.

Species specific oligo-nucleotides were used as the primer for molecular characterization of the isolated pathogen (*Alternaria spp.*) as per the protocol described by Tsai *et al*. (2006) with slight modifications.

To amplify the *Alternaria* species isolated from affected fruits, a set of species-specific oligo-nucleotide primers were selected. The genomic DNA was utilized as a template for amplification after being diluted to 1:9 amount of DNA and nuclease-free water. In a 0.5 ml PCR tube, 4 µl PCR master mix, 4.5 µl molecular grade distilled water, 0.50 µl (0.25 µl forward + 0.25 µl reverse) primer, and 1 µl template DNA were used in the PCR process. To avoid chemicals sticking to the wall, the reaction mixture was vortexed completely and briefly centrifuged.

The temperature profiles for PCR amplification were 95°C for 3 minutes of initial denaturation, followed by 30 cycles of denaturation at 95°C for 30 seconds, continuous annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 7 minutes. The products were kept at 4°C until the gel electrophoresis was completed after the polymerase chain reaction.

3.3.4 Separation of amplified products by Agarose gel electrophoresis:

The amplified PCR products were separated using an agarose gel electrophoresis with a 1 per cent agarose gel. The agarose gel was prepared and cooled to 60°C before adding 5 µl of ethidium bromide. The prepared gel solution was poured into the platform after the comb was put into the gel casting platform. While pouring, extra caution was taken to avoid trapping air bubbles in the gel. After allowing the gel to solidify, the comb was removed and the gel was placed into an electrophoresis apparatus containing enough 1X TAE buffer to completely cover the wells. The 5 µl (3 µl loading dye + 2 µl amplified product) were then carefully injected into sample wells with a control of 100 bp DNA ladder. The tracking dye was migrated to the end of the gel by electrophoresis at 60 V. The DNA bands stained with ethidium bromide were viewed using the Gel documentation system.

3.3.5 Morpho- cultural studies

Growth characters on solid media

The growth and cultural characters of *A. alternata* were studied on different media viz. Potato dextrose agar, Malt extract agar, Sabouraud's medium, Richard`s agar, Czapeck's agar, Oat meal agar and Mathur agar.

Each Petri plate contained fifteen ml of each medium, which was allowed to solidify. Such plates were injected with 5 mm discs of the test pathogen under study cut

from the culture's periphery and cultured for 12 days at room temperature (28 ±1°C). Each treatment was replicated three times. The colony diameter of each plate was calculated by averaging the linear colony growth in two directions. Color of the colony, elevation of the surface and sporulation were also recorded. The data on radial growth was statistically examined. The composition and preparation of the above mentioned synthetic and semi-synthetic media were obtained from Ainsworth and Bisby's 'Dictionary of the Fungi by Hawksworth *et al*. (1983).

3.3.6 *In vitro* evaluation of bioagents by dual culture technique.

The dual culture technique was used to test the antifungal activity of bioagents. The different bioagents were tested against selected disease causing fungi. The pathogen and bioagents were grown on different media for a week at 25 ± 2^0C. 5mm disc of the target fungi cut from the periphery was transferred to the Petri dish previously poured with sterilized potato dextrose agar media. Bioagents were transferred aseptically in the same plate of opposite end and incubated at room temperature with alternate light and darkness for 7 days and observed periodically. Control plates were also maintained without bioagent.

Effects of biocontrol agents on mycelial growth (mm) of Pathogen

Design : CRD

Treatments : Eight

Replications : Three

Method : Dual Culture Technique

Treatment Details:

Treatment	Bioagent
T_1	*Trichoderma asparallum*
T_2	*Trichoderma harzianum*
T_3	*Trichoderma hamatum*
T_4	*Aspergillus niger*
T_5	*Bacillus subtilis*
T_6	*Pseudomonas fluorescens*
T_7	*Metarhizium anisopliae*
T_8	Control

Mycelial growth of test pathogen were observed periodically after 24, 48, 72 and 96 hours and final observations were recorded when the control plate reached full growth in Petri plate. Per cent inhibition over control were calculated by using formula given by Vincent (1927),

$$\textbf{Per cent inhibition} = \frac{C - T}{C} \times 100$$

Where,

C= Mycelial growth in control plate

T= Mycelial growth in treated plate

3.3.7 *In vitro* evaluation of essential oils by poisoned food technique:

Essential oils which were reported effective against *Alternaria* spp. and were evaluated *in-vitro* by applying Poisoned Food Technique (Nene and Thapliyal, 1993) and using Potato dextrose agar as basal medium. The test pathogens were grown on PDA medium in Petriplates for fifteen days prior to setting the experiment. Essential oil suspension was prepared in PDA by adding required quantity of essential oil along with Twin-80 to obtain the desired concentration of essential oil. Twenty ml of poisoned medium was poured in each of the sterilized Petriplates. For this 20 ml of sterilized and cooled medium (PDA) was poured in each petriplate (90 mm diameter) and was allowed to solidify. A 5 mm disc of test pathogen was placed at centre of the medium with the help of sterilized cork borer. For this a week old culture of test pathogens in petridishes on sterilized PDA medium were used. Three replications for test pathogens and control i.e. without addition of any essential oil were maintained. Petriplates were incubated at 28 ± 2^0C temperature in inverted position. Observations on radial mycelial growth and per cent inhibition of the test fungus were recorded at 24 hrs interval and continued till growth of the test pathogen in untreated control plate is fully covered. Per cent inhibition of the test pathogen was calculated by applying formula given by Vincent (1927) as follows.

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

Where,

C = Growth of the test fungus in untreated control plates

T = Growth of the test fungus in treated plates

Treatment Details:

Design : CRD

Treatments : Eight

Replications : Three

Method : Poisoned food technique

***In vitro* evaluation of essential oils**

Treatments	Essential Oils	Rate of Application
T_1	Lemon (*Citrus limonum*)	2500 ppm , 3000 ppm
T_2	Eucalyptus *(Eucalyptus globulus)*	2500 ppm , 3000 ppm
T_3	Rose (*Rosa damascena*)	2500 ppm , 3000 ppm
T_4	Ginger (*Zingiber officinale*)	2500 ppm , 3000 ppm
T_5	Clove (*Syzygium aromaticum*)	2500 ppm , 3000 ppm
T_6	Citronella (*Cymbopogon nardus*)	2500 ppm , 3000 ppm
T_7	Cardamom (*Elettaria cardamomum*)	2500 ppm , 3000 ppm
T_8	Control	

3.3.8 *In vitro* evaluation of plant extracts by poisoned food technique:

Plant extracts which were reported effective against *Alternaria* spp. and were evaluated *in-vitro* by applying Poisoned Food Technique (Nene and Thapliyal, 1993) and using Potato dextrose agar as basal medium. The test pathogens were grown on PDA medium in Petriplates for fifteen days prior to setting the experiment. Plant extracts suspension was prepared in PDA by adding required quantity of plant extracts along with Twin-80 to obtain the desired concentration of extract. Twenty ml of poisoned medium was poured in each of the sterilized Petriplates. For this 20 ml of sterilized and cooled medium (PDA) was poured in each petriplate (90 mm diameter) and was allowed to solidify. A 5 mm disc of test pathogen was placed at centre of the medium with the help of sterilized cork borer. For this a week old culture of test pathogens in petridishes on sterilized PDA medium were used. Three replications for test pathogens and control i.e. without addition of any plant extracts were maintained. Petriplates were incubated at 28 ±

2^0C temperature in inverted position. Observations on radial mycelial growth and per cent inhibition of the test fungus were recorded at 24 hrs interval and continued till growth of the test pathogen in untreated control plate is fully covered. Per cent inhibition of the test pathogen was calculated by applying formula given by Vincent (1927) as follows.

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

Where,

C = Growth of the test fungus in untreated control plates

T = Growth of the test fungus in treated plates

Treatment Details:

Design : CRD

Treatments : Eight

Replications : Three

Method : poisoned food technique

***In vitro* evaluation of plant extracts:**

Treatments	Essential Oils	Rate of Application
T_1	Lemon (*Citrus limonum*)	2500 ppm , 3000 ppm
T_2	Eucalyptus *(Eucalyptus globulus)*	2500 ppm , 3000 ppm
T_3	Rose (*Rosa damascena*)	2500 ppm , 3000 ppm
T_4	Ginger (*Zingiber officinale*)	2500 ppm , 3000 ppm
T_5	Clove (*Syzygium aromaticum*)	2500 ppm , 3000 ppm
T_6	Citronella (*Cymbopogon nardus*)	2500 ppm , 3000 ppm
T_7	Cardamom (*Elettaria cardamomum*)	2500 ppm , 3000 ppm
T_8	Control	

3.4 Statistical analysis

The data obtained in all the experiments were statistically analyzed . The percentage values were transformed into arcsine values. The standard error (SE) and critical difference (C.D.) at level P = 0.01 were worked out and results obtained were compared statistically. All the statistical analysis was done using VNMKV-STAT statistical programme at Central Computer Laboratory, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani.

CHAPTER - IV

RESULTS AND DISCUSSION

CHAPTER-IV
RESULTS AND DISCUSSION

Present studies on management of heart rot of Pomegranate caused by *Alternaria alternata* were undertaken during, 2020-2021 on various aspects *viz*., symptomatology, isolation, identification of pathogen, pathogenicity test, variability (cultural, morphological and molecular), *in vitro* efficacy of different phytoextracts, essential oils and bioagents against the pathogen. The results obtained on all these aspects are being interpreted and discussed under following sub-heads.

4.1. Symptomatology in field (visual examination)

Symptoms of the heart rot disease in pomegranate in natural field at various locations as well as pathogenicity test carried out at Department of Plant Pathology showed typical symptoms induced by *Alternaria alternata* (Table-4.1 and Plate-4.1). Initially on foliage small, oval, discoloured, scattered irregular appearance of lesions were observed which later on turned brown to grey with yellow zone around the spots. It was also observed that the spot developed concentric rings and a target board appearance was found on the upper surface of leaves. When the spots were matured, they have dry, grey to brown centers with yellow concentric ring. Pomegranate heart rot or black heart damage was found to begin with aril decay with or without external symptoms on the surface of fruits. The symptoms were studied on different cultivars of pomegranate and the results so obtained are presented in Table 4.1 and Plate 4.1.

Eight samples showing typical symptoms of the disease were collected from eight different locations. Every sample showed symptoms of *A. alternata* but they varied minutely with the location of the samples collected. The pomegranate sample of Bhagwa cultivar collected from Mangalwedha tehsil of Solapur district had light to dark brown spots over the leaf surface, which were oval to irregular in shape. Similarly, the sample collected from Kanpur region of Uttar Pradesh showed initial brown, small, scattered spots which later coalesced to form larger spots which were circular with brown to black lesions. The disease samples of Bhagwa cultivar collected from Nanded district exhibited reddish brown spots which were small and circular in appearance. Ganesh cultivar obtained from Madha tehsil of Solapur district showed small, irregular, brownish spots on the leaf blades and margins, which enlarged to form elongated lesions on the leaf surface. Similarly, the Bhagwa sample

obtained from Beed district had infected leaves that turned rigid and their colour was changed to brown due to the disease infection. The Ganesh cultivar infected with the disease and obtained from Sangola tehsil showed brown to black colouration along with the disease symptoms, whereas, Ganesh cultivar of Parbhani district had small, circular reddish brown spots on the leaves. The Super Bhagwa cultivar obtained from NRCP, Solapur exhibited no external symptoms on the fruit surface but the fruits were found to turn light in colour due to internal decay.

Symptoms observed during present studies were matched with the symptoms observed by Pala *et al*. (2009), Ezra *et al*. (2010), Gat *et al.* (2012), Khosla and Bhardwaj (2013), Ammar and El-Naggar (2014), Ezra *et al.* (2015), Gaikwad and Karande (2016) and Kahramanoglu *et al.* (2018). Pala *et al.* (2009) reported that *A. alternata* which induced fruit rot in pomegranate showed symptoms on leaves, flowers and young fruits. Initially uneven small dark lesions were coalesced and covered half portion of leaves and then the flowers and small fruits were found infected. Kahramanoglu *et al.* (2018) reported that heart rot or black heart damage in pomegranate started as aril decay with no external symptoms. But infected fruits showed unusual skin colour and abnormal shapes which leads to reduced marketable quality.

4.1.1 Isolation of different *Alternaria* spp. from naturally infected Pomegranate plants.

The diseased affected Pomegranate plants were identified in natural conditions based on key symptoms of leaf spot and heart rot disease. These samples were brought in laboratory and applying the tissue isolation method, *Alternaria alternata* were isolated on potato dextrose agar (PDA) medium. These cultures were categorized as ALT-1 to ALT-8. The results obtained with regard to isolation of *Alternaria alternata* and colony characters of all isolates is presented in Table 4.2.

It was observed that the different isolates collected from different locations produced initially white mycelium which was hyaline when observed under microscope. These colonies were turned to black colour in advanced stage.

The colony colour of above mentioned isolates were found varied, where, ALT-1 and ALT-6 showed black coloured colonies, whereas, ALT-2, ALT-7, ALT-8 showed greyish black appearance. The isolate ALT-3, ALT-4 and ALT-5 were olivacious black, off white and creamy white colonies, respectively.

Table: 4.1 Symptomatology of *Alternaria alternata* on various cultivars of Pomegranate.

Sr. No.	Location of sample collected			Cultivar/Variety	Typical symptoms observed
	Name of Farmer/Place	Tq.	District		
1	Samlinga Sangolkar	Mangalwedha	Solapur	Bhagwa	Light to dark brown, roundish-oval to irregular spots on leaves.
2	Subodh Agarwal	Kanpur	Kanpur	Bhagwa	On leaves initially small, brown, scattered dots, which later enlarged and coalesced to form large, circular or irregular brown to black lesions.
3	Maruti Katare	Nanded	Nanded	Bhagwa	Small, circular and light to reddish brown spots on leaves.
4	Vitthal Nagtilak	Madha	Solapur	Ganesh	Initially small, irregular, brown spots on leaf blades and margin which later enlarged and coalesced forming elongated lesions
5	Pratap Chavan	Beed	Beed	Bhagwa	Infected leaves turned rigid and their colour changed from yellowish brown to dark brown.
6	Prakash Vibhute	Sangola	Solapur	Ganesh	On leaf margin brown to black colouration.
7	VRS farm, VNMKV Parbhani	Parbhani	Parbhani	Ganesh	Initially on leaves small, circular and light to reddish brown spots found.
8	Plot No. H-34 NRCP, Solapur	NRCP Solapur	Solapur	Super Bhagwa	No external symptoms appeared but fruit became light in colour and rind turned slightly off coloured due to internal decay

The colony appearance of the test isolates ALT-4 and ALT-7 was fluffy, whereas it was feathery in case of ALT-2, ALT-5 and ALT-6. The remaining isolates i.e. ALT-1, ALT-3 and ALT-8 showed cottony appearance of the colony.

Based on growth rate, measured12 days after inoculation that the test isolates were grouped as fast growing, moderately growing and slow growing. Among these isolates, ALT-1, ALT-3, ALT-4, ALT-7 and ALT-8 were found to grow very fast as compared to other isolates, whereas, ALT-5 and ALT-6 were moderate in growth. Among all these isolates, ALT-2 showed very slow growing pattern.

Shape of the colony was circular with smooth margin in five isolates i.e. ALT-1, ALT-3, ALT-4, ALT-7 and ALT-8 and irregular with rough margin in rest of the three isolates i.e. ALT-2, ALT-5 and ALT-6.

The sporulation of the isolates was varied from good to excellent sporulation behaviour. The isolates ALT-1, ALT-3, ALT-5 and ALT-8 showed excellent sporulation, whereas, ALT-4 and ALT-6 showed good sporulation as well as ALT-2 and ALT-7 showed fair sporulation.

The observations of the present studies are found similar with the reports of earlier researchers where Keissler (1912) described morphology of *A. alternata* as black or olivaceous black and sometimes grey colonies. Conidiophores were produced singly, simple or branched, straight or flexuous, sometimes geniculate, pale to mid olivaceous or brown, smooth, measuring 50 µm long, 3-6 µm thick. Conidia formed in long, branched chains, obclavate, pyriform, ovoid or ellipsoidal often with short conical or cylindrical beak, not more than one third length of the conidium, pale to mild golden brown, smooth or verrucose with eight transverse and usually several longitudinal or oblique septa. Mangala *et al*. (2006) stated the morphological characters of *A. alternata* causing leaf blight of chilli. It was reported that fungal colonies were dull white coloured initially then turned olive green upon sporulation from fifth day. The conidia were observed as obclavate with beak, thorny and pigmented with relatively 1-9 horizontal septa and 0-4 vertical septa and measured about 45.77- 47.85 µm in breadth.

Plate 4.1. Symptoms observed on leaf and fruit sample of Pomegranate

Table 4.2 Morphological characters of different isolates of *Alternaria alteranata*

Characters	Isolates (12DAI)							
	Alt-1	**Alt-2**	**Alt-3**	**Alt-4**	**Alt-5**	**Alt-6**	**Alt-7**	**Alt-8**
Colony Dia. (mm)	90.00	68.14	90.00	90.00	72.64	67.02	88.11	90.00
Colony Colour	Black	Grayish black	Olivaceous black	Off white	Creamy white	Black	Grayish black	Grayish black
Appearance	Cottony	Feathery	Cottony	Fluppy	Feathery	Feathery	Fluppy	Cottony
Growth rate	Fast	Slow	Fast	Fast	Moderate	Moderate	Fast	Fast
Shape	Circular	Irregular	Circular	Circular	Irregular	Irregular	Circular	Circular
Margin	Smooth	rough	smooth	smooth	rough	rough	smooth	smooth
Sporulation	++++	++	++++	+++	++++	+++	++	++++

*Sporulation : ++++ = Excellent, +++ = Good, ++ = Fair, + = Poor, Dia : Diameter.

4.3 Identification of the pathogens

Based on the typical symptoms, morphological and cultural characteristics, microscopic observations and pathogenicity test, the pomegranate heart rot pathogen was identified and confirmed as *Alternaria alternata* (Table-4.2). This pathogen was identified according to morphological identification based on characteristics of the conidia and colony growth characters. The identification of the culture was further confirmed by the presence of conidia (Ellis 1971). Pure cultures of all the isolates were maintained and stored on PDA for further studies.

The observations of present studies are in full agreement with the report of earlier worker. Ezra *et al*. (2010) isolated *Alternaria* spp. from infected pomegranate fruits and identified as *Alternaria alternata* based on fungal colonies emerged from symptomatic tissue which had morphology and conidia typical to *Alternaria* spp. Sequence analysis of rDNA ITS region and β-tubulin gene of four different isolates exhibited 100 per cent identity to *Alternaria alternata*. Similarly, Ezra *et al*. (2015) isolated fungus from both soft and dry rotted arils and identified as species of *Alternaria*. Several single spore *Alternaria* culture were examined through sequencing of ITS 5.8 S rDNA. All the isolates used in the study exhibited 99 per cent identity to sequences of *A. alternata*.

4.4. Molecular identification and characterization of *Alternaria* spp.

In the present study, species specific primers were used for the identification of isolated pathogen at species level. The results of polymerease chain reaction (PCR) and gel documentation ensured the specificity of the designed primers. (Plate 4.3)

Set of species specific primers ITS1/ITS4 for *Alternaria alternata* were used to identify the eight different isolates collected from different locations. The expected length i.e 540 bp of all DNA fragments were obtained when polymerase chain reaction (PCR) product primed with ITS1/ITS4. Thus the results confirmed that all isolates were of *Alternaria alternata*.

The observations of present studies are in full agreement with the report of earlier worker. Abeer *et al.* (2014) extracted DNA samples from mangrove leaves, healthy as well as artificially infected with *A. alternata*. Seven forward and reverse primers were used in this study. PCR amplification with primer Al-f1/Al-r1 expressed 370 bp amplification product in both *A. alternata* isolates and the artificially infected

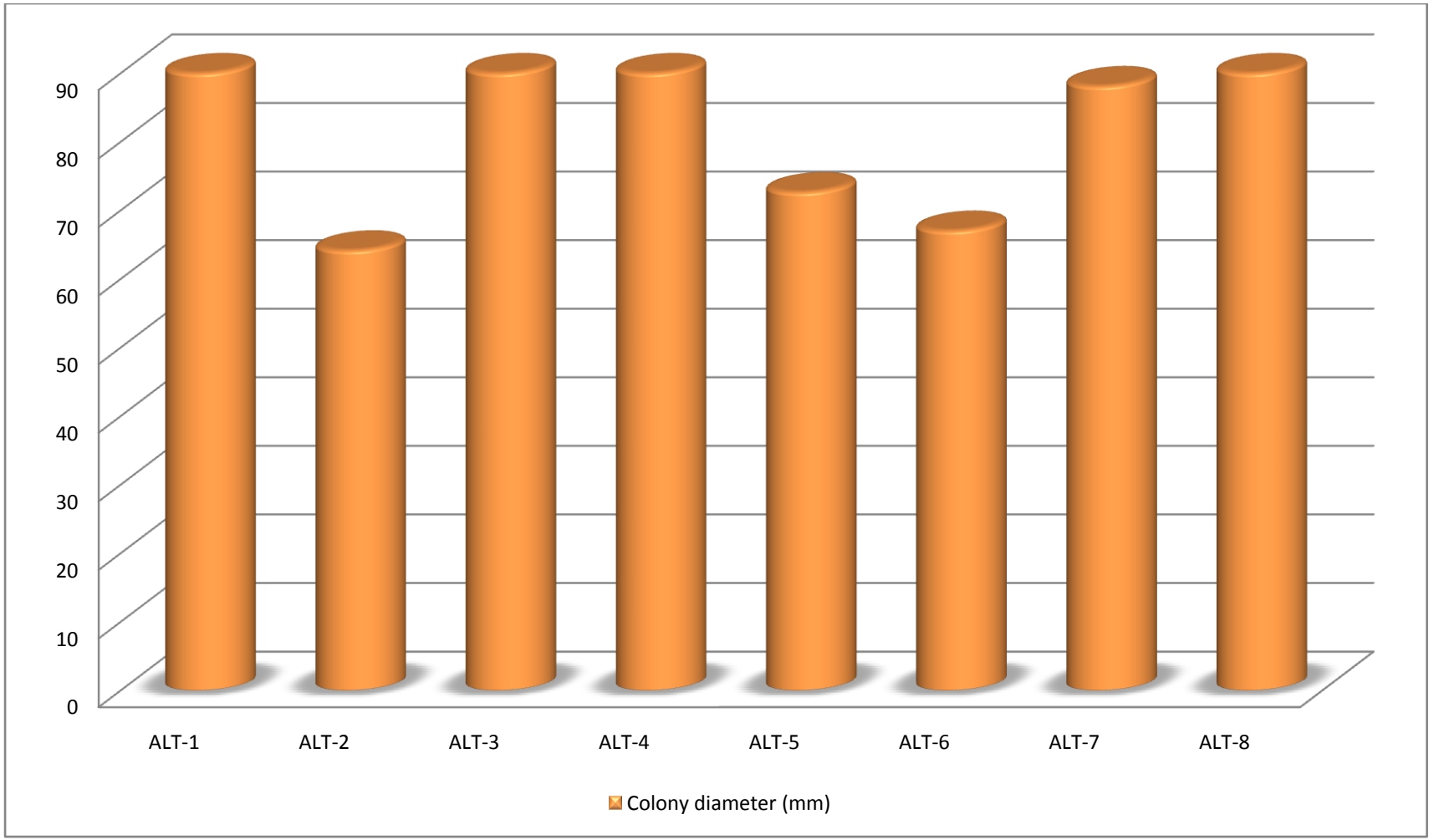
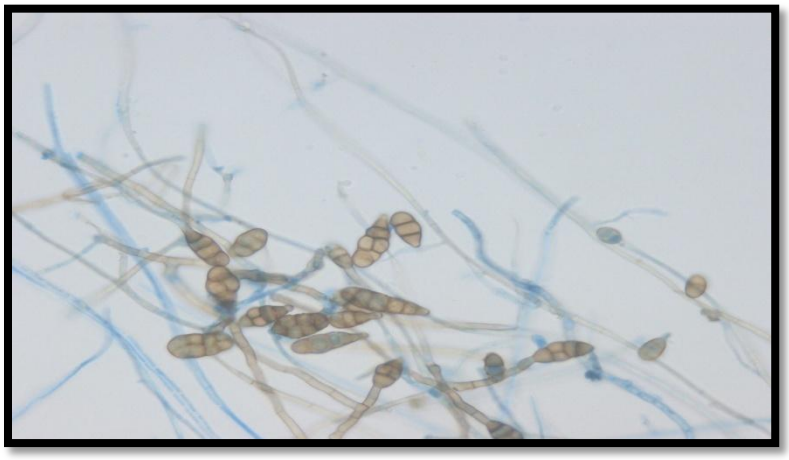
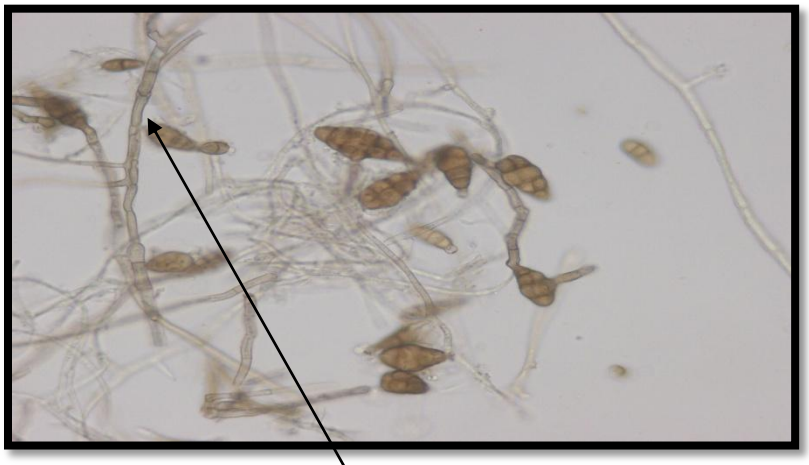


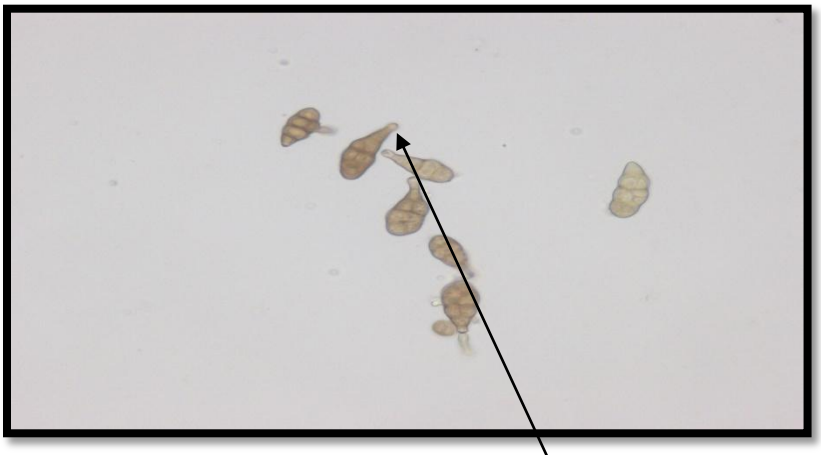
Fig.4.1: Morphological characters of different isolates of Alternaria alteranata



Conidia

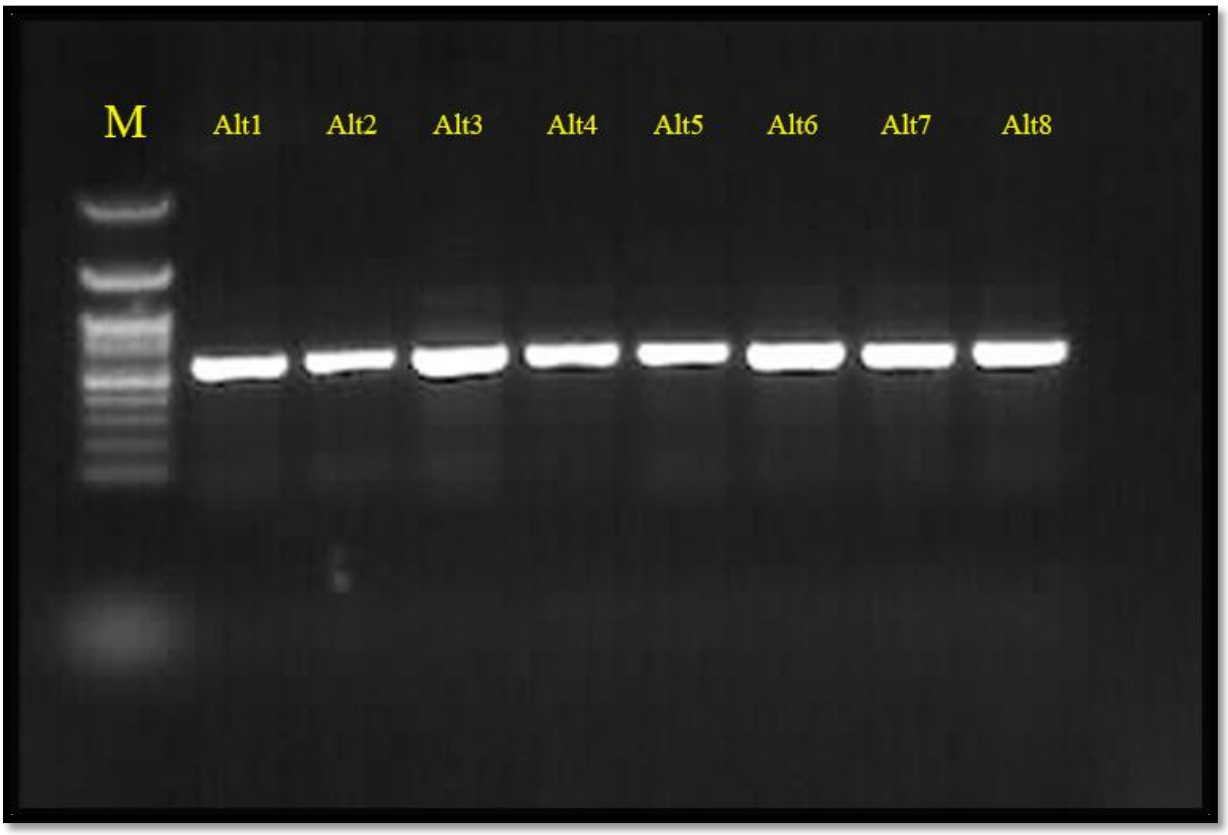


Septate mycelium



Conidial beak

Plate 4.2. Microphotograph of the mycelium and spores of *Alternaria alternata*



M: Marker (100 bp Ladder)

Alt1:*Alternaria alternata* Mangalwedha isolate

Alt2: *Alternaria alternata* Kanpur isolate

Alt3: *Alternaria alternata* Nanded isolate

Alt4: *Alternaria alternata* Madha isolate

Alt5: *Alternaria alternata* Beed isolate

Alt6: *Alternaria alternata* Sangola isolate

Alt7: *Alternaria alternata* Parbhani isolate

Alt8: *Alternaria alternata* NRCP Solapur isolate

Plate 4.3. Amplification of *Alternaria alternata* with ITS1/ITS4 primers.

mangrove leaves only, whereas, no amplified product was detected in case of healthy mangrove leaves with the diagnostic primer pair (Al-f1/Al-r1).

4.5. Pathogenicity test

Pathogenicity test was attempted by spraying the spore suspension (2×10^6 cfu/ml) of *Alternaria alternata* on healthy growing, one month old seedlings of pomegranate cultivar Super Bhagwa. The seedlings were incubated in the screen house, where relative humidity (80%) and optimum temperature (28 ± 2^0 C) were maintained for the further development of symptoms. After two weeks of incubation, typical symptoms *Alternaria* leaf spot on foliage of artificially inoculated pomegranate seedlings were observed 14 days after inoculation, however, the control plant sprayed with only sterilized distilled water remained healthy and did not produce any kind of symptoms throughout the period of observation.

Similarly, healthy mature fruits were also inoculated with pathogen in laboratory and placed in moist chamber for development of symptoms. The symptoms of heart rot disease were appeared after five days of inoculation. The re-isolation of *A. alteranata* was carried out from artificially inoculated leaves as well as fruits, which confirmed the association of same pathogen with the host. The results so obtained revealed that *A. alternata* was found pathogenic to pomegranate, causing *Alternaria* leaf spot and heart rot (plate 4.4 and 4.5).

The results of the present studies are in conformity with the results of previous workers *viz.* Trizos *et al*. (2008), Ezra *et al*. (2010), Gat *et al*. (2012) and Kumar *et al*. (2017). Ezra *et al.* (2010) conducted an experiment to justify the pathogen causing black spot of pomegranate as *A. alternata* by detached fruit and leaf method. Gat *et al*. (2012) confirmed the causal agent for the black spot of pomegranate as *A. alternata* on detached leaves, fruits and flower by artificial inoculation of fungal spore suspension. Pomegranate seedlings were also used to prove the pathogenicity of *A. alternata* causing pomegranate heart rot by following detached fruit technique.

4.6.Morpho-Cultural variability.

Results presented in Table 4.3 and 4.4 revealed that all the eight test isolates of *A. alternata* exhibited a wide range of morphological variability such as mycelial width, conidial dimensions, beak length of conidia and numbers of vertical and horizontal septa.

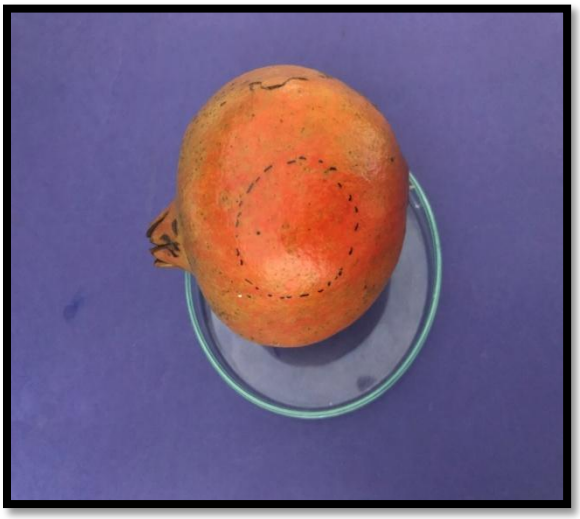
On the basis of mycelial width, the test isolates were categorized into three groups *viz*., large, medium and small sized. Two isolates i.e ALT-7 and ALT-8 were observed as large sized, of which mycelial width was 8.15 and 8.20 µm in ALT-7 and ALT-8, respectively. Three isolates (ALT-1, ALT-3 and ALT-5) showed medium sized mycelial width in the range of 6.25-7.20 µm. However, it was maximum in ALT-3 (7.20 µm), followed by ALT-5 (6.78 µm) and ALT-1 (6.25 µm). Rest of the three isolates (ALT-2, ALT-4 and ALT-6) showed small sized mycelial width in the range of 4.48-5.55µm. However, it was maximum in ALT -4 (5.55 µm), followed by ALT -6 (5.25 µm), ALT -2 (4.48 µm).

Based on length and breadth of the conidia, the test isolates were grouped as large, medium and small sized conidia. One isolate i.e ALT-4 exhibited large sized conidia (48.21 L X 7.25 W), whereas, three isolates i.e. ALT-3, ALT-7 and ALT-8 exhibited medium sized conidia (42.23-47.22 L X 8.23-8.74 W µm), however, it was maximum in ALT-3 (47.22 X 8.74µm), followed by ALT-7 (42.39 X 9.82µm) and ALT-8 (42.23 X 8.23µm). Rest of the four isolates (ALT-1, ALT-2, ALT-5 and ALT-6) exhibited small sized conidia (39.43 -40.48 L X 5.45-6.88 W µm).

Three isolates (ALT-2, ALT-3 and ALT-7) showed medium sized beak length (9.15-12.15µm), however, it was highest in ALT -7 (12.15 µm), followed by ALT-3 (11.22µm) and ALT-2 (9.15µm). Rest of the three isolates (ALT-1, ALT-4 and ALT-6) exhibited small sized beak length (8.51-9.00 µm).

In all of the test isolates, horizontal septation was ranged from 1-6 and vertical septation from 1-5.

The results of the present studies are in conformity with the results of previous workers. Keissler (1912) described morphology of *A. alternata* as black or olivaceous black and sometimes grey colonies. Conidiophores were produced singly, simple or branched, straight or flexuous, sometimes geniculate, pale to mid olivaceous or brown, smooth, measuring 50 µm long, 3-6 µm thick. Conidia formed in long, branched chains, obclavate, pyriform, ovoid or ellipsoidal often with short conical or cylindrical beak, not more than one third length of the conidium, pale to mild golden brown, smooth or curved with eight transverse and usually several longitudinal or oblique septa.



Healthy fruit

Inoculated fruit

Symptoms on inoculated fruit

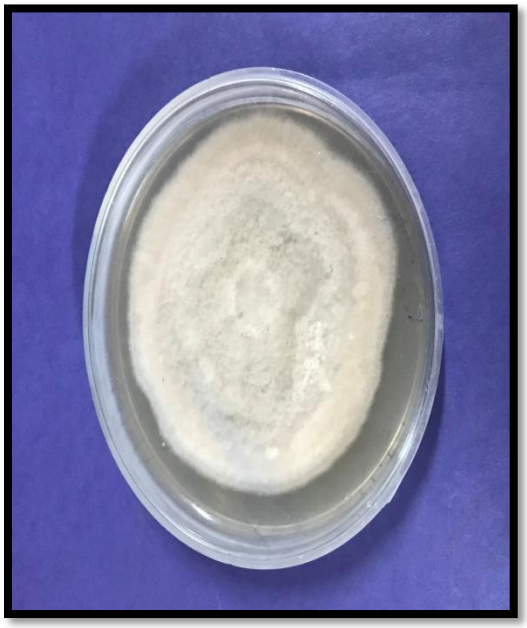
Re-isolated pathogen

Plate 4.4. Pathogenicity of *Alternaria alternata* on fruits. (Detached fruit method).

Healthy Seedling

Inoculation of spore

Symptoms on inoculated seedling



Re-isolated pathogen

Plate 4.5. Pathogenicity of *Alternaria alternata* on seedlings.

4.6. Effect of different culture media

Table 4. 3 Morphological variability among the isolates of *A.alternata.*

Sr.No	Isolates	Av. Mycelial width (µm)	Av. Size of Conidia (L X W µm)	Av. Beak length (µm)	Number of Septa (Range)	
					H	V
1	ALT-1	6.25	39.43 X 5.45	8.51	1-5	1-2
2	ALT-2	4.48	40.28 X 6.52	9.15	2-6	1-4
3	ALT-3	7.20	47.22 X 8.74	11.22	1-4	1-3
4	ALT-4	5.55	48.21 X 7.25	8.81	3-5	1-2
5	ALT-5	6.78	40.48 X 6.88	15.26	2-5	1-3
6	ALT-6	5.25	39.98 X 6.07	9.00	3-5	1-2
7	ALT-7	8.15	42.39 X 9.82	12.15	2-5	1-2
8	ALT-8	8.20	42.23 X 8.23	13.49	3-5	1-5

H : Horizontal, V : Vertical

Table 4.4 Grouping of *A. alternata* isolates based on morphological variability.

Sr.No	Conidia	Size (µm)	No. of Isolates	Isolate Code
I. Mycelial width				
1.	Small	4.48-5.55	3	ALT-2, ALT-4 , ALT-6
2.	Medium	6.25-7.20	3	ALT-1, ALT-3, ALT-5
3.	Large	8.15-8.20	2	ALT-7, ALT-8
II. Conidia size (LX W µm)				
1.	Small	39.43 X 5.45-40.48 X 6.88	4	ALT-1, ALT-2, ALT-5, ALT-6
2.	Medium	42.23 X 8.23-47.22 X 8.74	3	ALT-3, ALT-7, ALT-8
3.	Large	48.21 X 7.25 and above	1	ALT-4
III. Beak Length				
1.	Small	8.51-9.00	3	ALT-1, ALT-4, ALT-6
2.	Medium	9.15-12.15	3	ALT-2, ALT-3, ALT-7
3.	Large	13.49-15.26	2	ALT-5, ALT-8

Table 4.5 Effect of different culture media on growth characteristics of *A. alternata*

Sr. No	Solid media	Colony dia. of test pathogen * (mm)	Colony Characters		
			Colony growth and margin	Colony colour	Pigmentation
1	Richard's Agar	32.49	Flat and regular	Greyish Black	Black
2	Czapek's Dox Agar	72.97	Fluffy and irregular	Greyish black	Black
3	Malt extract agar	81.33	Fluffy and irregular	Grey to black	Black
4	Saboraud's Dextrose Agar	72.38	Fluffy and irregular	Light Grey	Blackish white
5	Oat Meal Agar	90.00	Fluffy and regular	Greyish black	Black
6	V8 Juice Agar	36.22	Fluffy and regular	Blackish grey	Black
7	Potato Dextrose Agar	48.33	Fluffy and Irregular	Light Grey	Light Grey
	SE ±	**0.28**			
	CD (P=0.01)	**1.21**			

* Mean of three replications, Dia.: Diameter

Cultural characteristics *viz.*, mycelial growth, colour of the colony and concentric rings produce by *Alternaria alternata* were studied *in vitro* using six synthetic and one non synthetic culture media. All the media tested encouraged better growth of the test pathogen. (Table 4.5, Fig 4.2 and Plate 4.6)

All the six synthetic culture media tested, encouraged better growth of *A. alternata* and exhibited mostly similar effects on cultural characteristics of the test pathogen. However, Oat meal agar was found most suitable and encouraged maximum radial mycelial growth (90.00 mm). The second best culture medium found was malt extract agar (81.33mm) which was followed by Czapek's Dox agar (72.97 mm) and Sabouraud's dextrose agar (72.38 mm). Rest of the culture media exhibited less growth amongst the media tested viz. Potato dextrose agar, V8 juice agar, and Richard's agar media showed the mycelia growth of 48.33 mm, 36.22 mm and 32.49 mm, respectively. All culture media tested encouraged dense mycelial growth, circular to irregular, white grey, pinkish white, dark brown, whitish red, light brown coloration of the colonies with concentric rings.

These results are in conformity with effect of different culture media on growth and cultural characteristics of *Alternaria alternata* reported earlier by several workers. Suryawanshi *et al.* (2010) reported that *A. alternata* inducing heart rot of pomegranate showed abundant growth and sporulation on Sabouraud's agar. Archana (2012) reported that among different media tested, the mean mycelial growth of *Alternaria alternata* was maximum in PDA and Sabourad's agar followed by Richards agar, whereas, sporulation was maximum in PDA and host leaf extract + 1% sucrose agar.

4.7 *In vitro* bioefficacy of bio agents against *Alternaria alternata.*

The results obtained on mycelial growth and inhibition of growth of *A. alternata* with five fungal and two bacterial antagonists are presented in Table 4.6 and Fig 4.3. The results revealed that all the bioagents exhibited fungistatic / antifungal activity against *A. alternata* and significantly inhibited its growth, over untreated control.

Among all the antagonists tested, *Trichoderma asperellum* was found most effective in inhibiting the pathogen. There was significant reduction in mycelial growth of *Alternaria alternata* by *T. asperellum* (16.18 mm) as compared to other

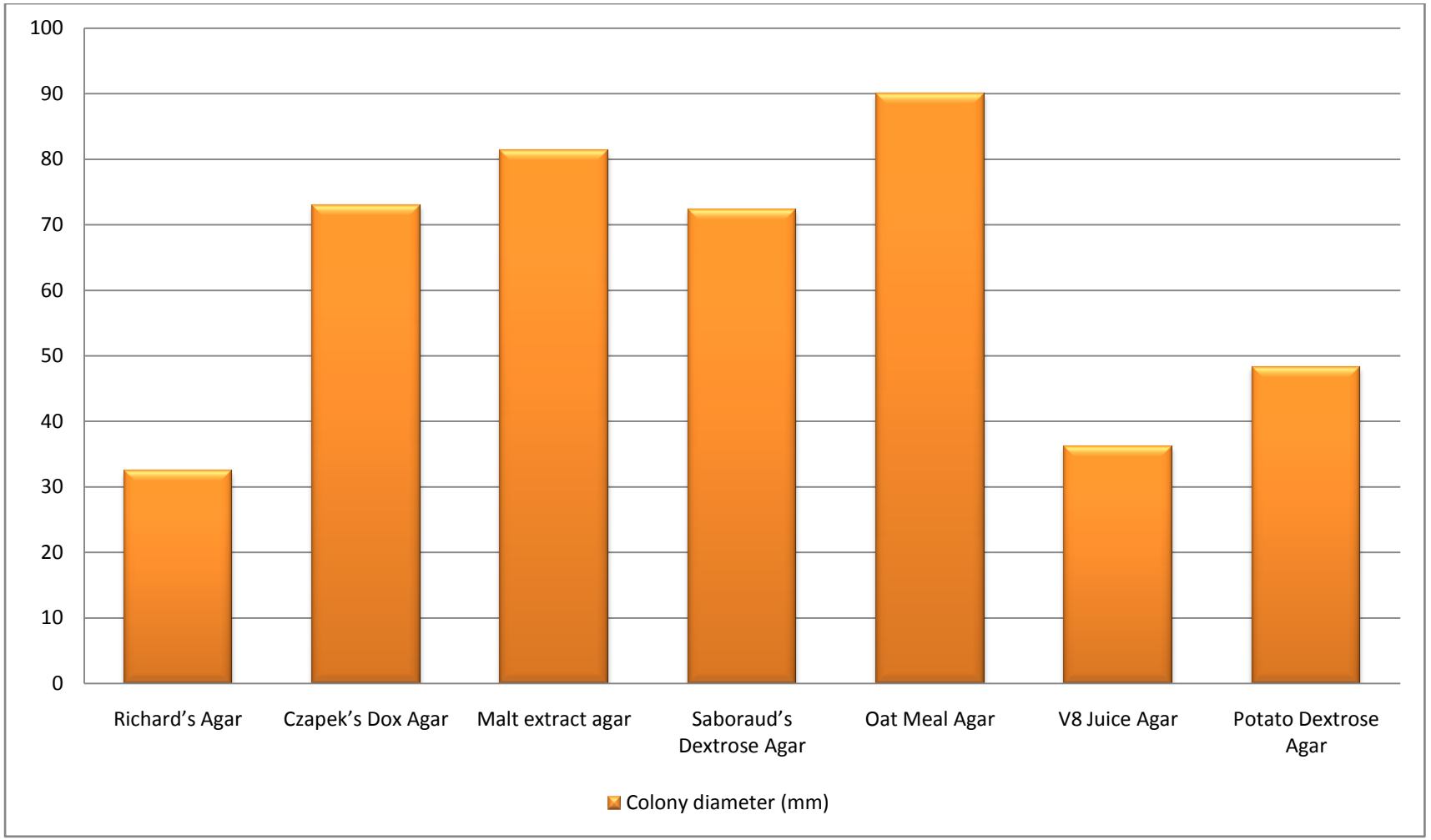
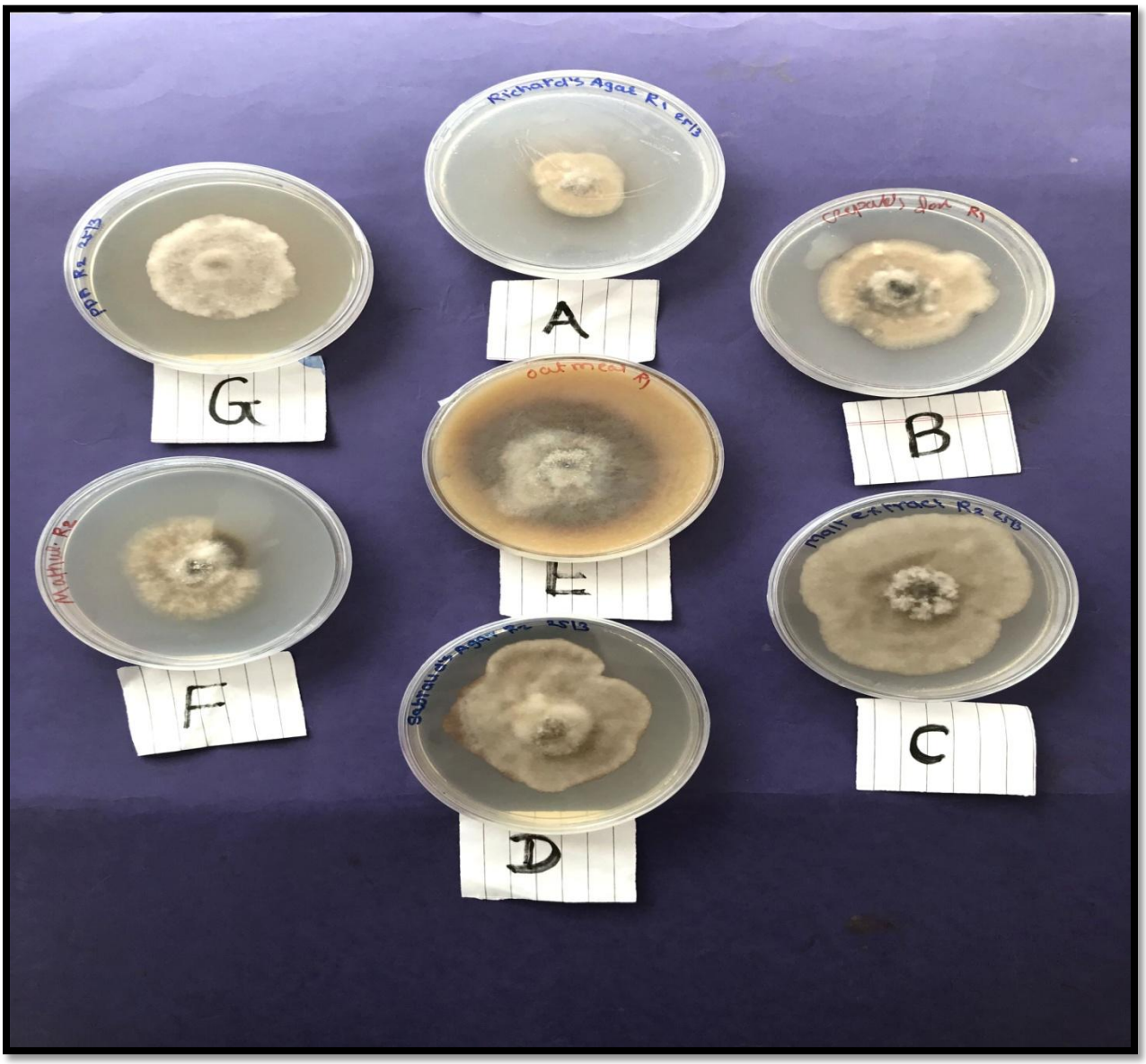


Fig.4.2: Effects of different culture media on growth of *Alternaria alternata.*



A- Richard's Agar
B- Czapek's Dox Agar
C- Malt extract Agar
D- Saboraud's Agar
E- Oat meal Agar
F- V8 juice
G- Potato Dextrose Agar

Plate 4.6 Effects of different culture media on growth of *Alternaria alternata*.

Table 4.6 In-vitro bioefficacy of different bioagents against *A. alternate*

Tr. No.	Treatments	Colony dia. of test pathogen * (mm)	Per cent inhibition
T1	*Trichoderma asperellum*	16.18	81.48 (64.51)
T2	*T. harzianum*	19.89	77.24 (61.50)
T3	*T. hamatum*	22.41	74.36 (59.57)
T4	*Aspergillus niger*	17.49	79.99 (63.42)
T5	*Bacillus subtilis*	48.63	44.36 (41.76)
T6	*Pseudomonas fluorescens*	43.37	50.38 (45.21)
T7	*Metarhizium anisoplae*	22.68	74.05 (59.37)
T8	Control (untreated)	87.41	00 (00)
	SE ±	**0.54**	**0.47**
	CD (P=0.01)	**2.25**	**1.98**

* Mean of three replications, Dia.: Diameter
Figures in parentheses are arcsine transformed values.

treatments. It was followed by *Aspergillus niger* (17.49 mm), *T. harzianum* (19.89 mm), *T. hamatum* (22.41 mm), *M. anisoplae* (22.68 mm), *Pseudomonas fluorescens* (43.37 mm) and *Bacillus subtilis* (48.63 mm). Maximum growth of *Alternaria alternata* was observed in control plate i.e. 87.41 mm (Plate 4.7).

Among the different antagonists tested, *T. asperellum* was found most significant with highest mycelial growth inhibition (81.48 %) of the test pathogen followed by *Aspergillus* niger (79.99 %), *T. harzianum* (77.24 %), *T. hamatum* (74.36 %), *M. anisoplae* (74.05 %) and *Pseudomonas fluorescens* (50.38 %). Among all bioagents tested least per cent inhibition of pathogen was observed in treatment of *Bacillus subtilis* (44.36 %).

These results of the present study were in consonance with the earlier findings of workers. Apet *et al.* (2014) evaluated various fungal bioagents *in vitro* against *A. alternata*, causing leaf spot of gerbera and reported that highest mycelial growth inhibition was observed with *T. viride*(86.67%), followed by *T. hamatum* (78.34%), *T. koningii*(76.67%), *T. lignorun*(68.15%), *T. harzianum*(53.16%) and *Pseudomonas fluorescens* (50.38%). Kadam *et al.* (2018) tested the efficacy of bio-agents and botanicals *in vitro* against *A. alternata* causing leaf spot of pomegranate. Among the tested bio agents *T. viride* recorded significantly highest mycelial growth inhibition (86.85 %) followed by *T. hamatum* (82.04 %) and *A. niger* (81.11 %). Also the similar results were found with Ghosh *et al*. (2002), Mumtaz *et al*. (2012), Apet *et al*,*(* 2014), Kadam *et al*.(2018) and Marchande *et al*. (2020).

4.8 *In vitro* efficacy of phytoextracts against *Alternaria alternata.*

Seven different phytoextracts were evaluated at 10 and 20 per cent concentration against *Alternaria alternata* by applying poisoned food technique and the results so obtained are presented in Table 4.7 and Fig 4.4. The results revealed that all the tested plant extracts exhibited a wide range of radial mycelial growth of *Alternaria alternata* over untreated control (46.00 mm) and was found to be decreased with the increased concentration of plant extracts tested (Plate 4.8).

At 10 per cent concentration of plant extracts, the mycelial growth of *Alternaria alternata* was observed in the range of 20.45 mm to 46.00 mm. The least growth of *Alternaria alternata* was observed in the treatment of Neem leaf extract (20.45 mm). The next best treatment followed by this was *Parthenium* leaf extract (22.69 mm), Turmeric leaf extract (24.60), Nilgiri leaf extract (25.04 mm), Karanj leaf extract (25.14 mm) and Periwinkle leaf extract (27.49 mm). Amongst all plant

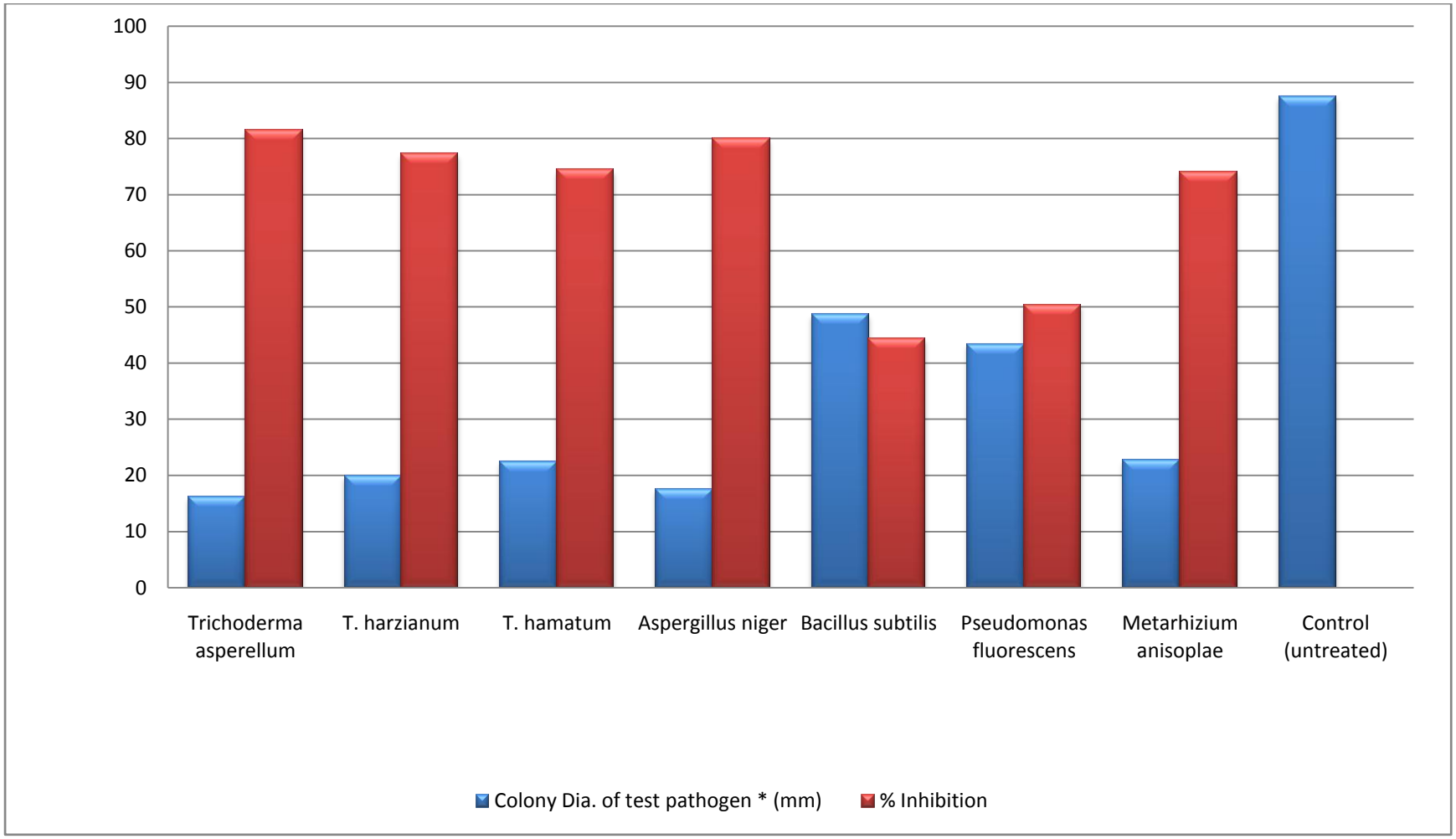
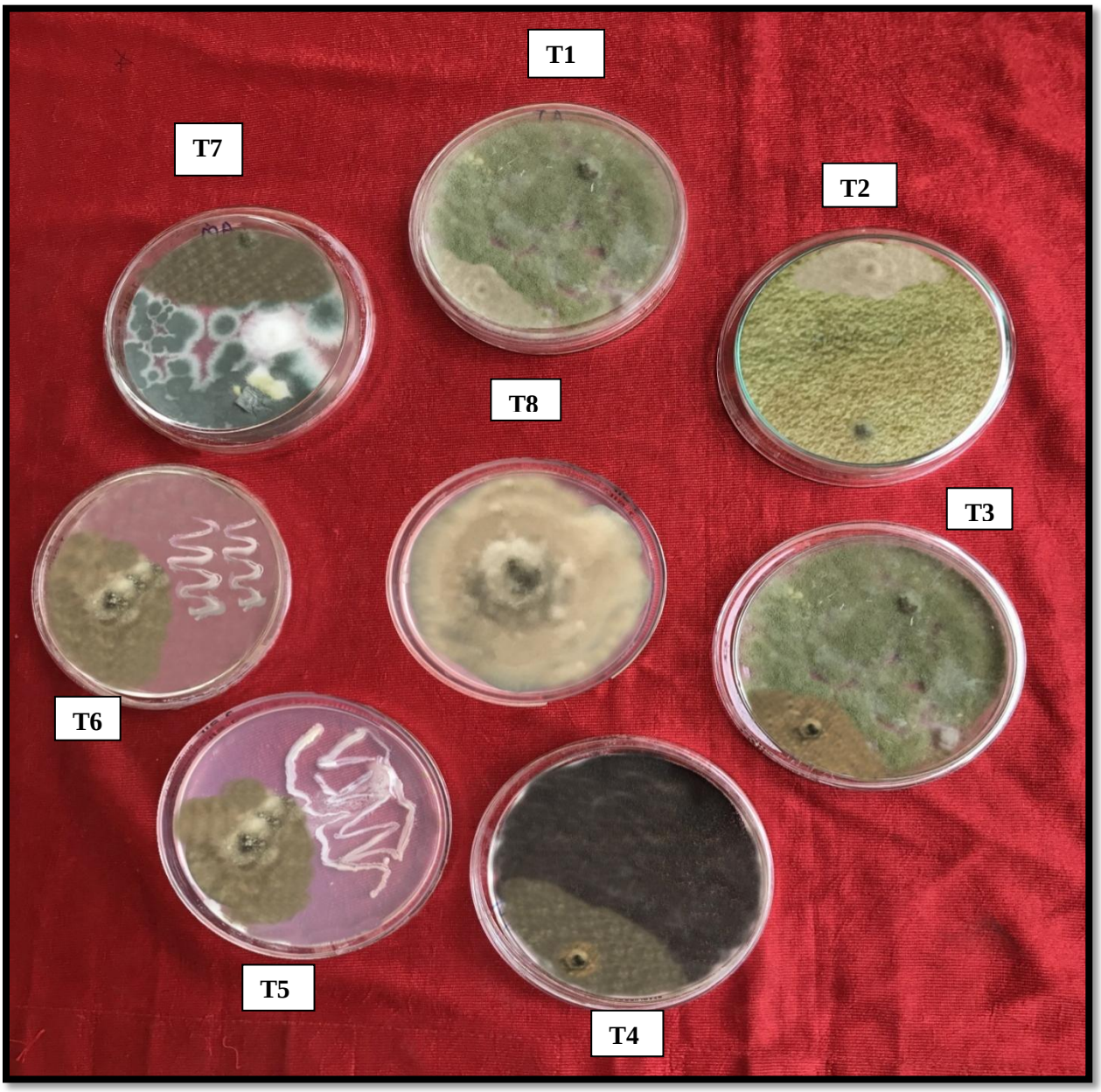


Fig.4.3: In vitro bioefficacy of bioagents against *Alternaria alternata.*



T1:*Trichoderma asperllum* **T2:***Trichoderma harzianum*

T3:*Trichoderma hamatum* **T4:** *Aspergillus niger*

T5:*Bacillus subtilis* **T6:***Pseudomonas fluorescens*

T7:*Metarhizium anisoplae* **T8:Control.**

Plate 4.7. *In vitro* bioefficacy of different bioagents against *Alternaria alternata.*

extract tested, Tulsi leaf extract was found comparatively less effective with maximum mycelial growth of pathogen i.e. 28.76 mm.

At 20 per cent concentration of plant extracts, the mycelial growth of *Alternaria alternata* was observed in the range of 20.52 mm to 46.00 mm. The least mycelia growth of *Alternaria alternata* was observed in the treatment of Neem leaf extract (20.52 mm) followed by *Parthenium* leaf extract (22.31 mm) and Turmeric leaf extract (22.61). The next treatments followed by this were Karanj leaf extract (22.78), Nilgiri leaf extract (24.74 mm) and Periwinkle leaf extract (25.77 mm). Amongst all plant extract tested, Tulsi leaf extract was found comparatively less effective with maximum mycelial growth of pathogen i.e. 26.89 mm.

Among the phytoextracts tested at 10 per cent concentration, Neem leaf extract was found most significant with highest mycelial growth inhibition of the test pathogen i.e 55.54 per cent, which was followed by *Parthenium* leaf extract (50.67%), Turmeric leaf extract (46.52%), Nilgiri leaf extract (45.56%), Karanj leaf extract (45.34%) and Periwinkle leaf extract (40.21%). Amongst all plant extracts Tulsi leaf extract was found to be less effective with minimum mycelia inhibition of pathogen i.e. 37.47 per cent.

Among the phytoextracts evaluated at 20 per cent concentration, Neem leaf extract was found most significant with highest mycelial growth inhibition of the test pathogen i.e. 53.74 per cent which was followed by *Parthenium* leaf extract (49.68%), Turmeric leaf extract (49.03%), Karanj leaf extract (48.64%), Nilgiri leaf extract (44.19%) and Periwinkle leaf extract (41.88%). Amongst all plant extracts, Tulsi leaf extract was found to be less effective with minimum mycelia inhibition of pathogen i.e. 39.35 per cent.

These results of the present study were in consonance with the earlier findings of Pareek *et al*. (2012), Bhosale *et al*. (2014), Rai *et al.* (2017) and Rajhans and Sharma. (2017). Bhosale *et al.* (2014) tested different aqueous leaf extract against *Alternaria alternata* of soybean. It was reported that neem, ginger and eucalyptus were found highly inhibitory to mycelial growth of *A. alternate.* Similarly Rai *et al*. (2017) evaluated three botanicals (Neem, Garlic and Onion) against *Alternaria* spp. inducing *Alternaria* leaf spot in cabbage. Results showed that Neem leaves extract at 5 per cent inhibited the development of *Alternaria* spp by 66.83 per cent followed by Onion bulbs extract at 4 per cent (38.12%) and Garlic bulb extract at 3 per cent (25.95%).

Table 4.7 *In-vitro* efficacy of different phytoextracts against *A. alternata*

Tr. No.	Treatments	Col. Dia		Per cent inhibition	
		10 %	**20%**	**10%**	**20%**
T1	Neem leaves extract	20.45	20.52	55.54 (48.18)	53.74 (47.14)
T2	Karanj leaves extract	25.14	22.78	45.34 (42.32)	48.64 (44.22)
T3	Periwinkle leaves extract	27.49	25.77	40.21 (39.35)	41.88 (40.32)
T4	Nilgiri leaves extract	25.04	24.74	45.56 (42.45)	44.19 (41.66)
T5	Turmeric leaves extract	24.60	22.61	46.52 (43.00)	49.03 (44.44)
T6	*Parthenium* leaves extract	22.69	22.31	50.67 (45.38)	49.66 (44.80)
T7	Tulsi leaves extract	28.76	26.89	37.47 (37.74)	39.35 (38.85)
T8	Control (untreated)	46.00	44.34	--	--
	SE ±	**0.61**	**0.54**	**0.57**	**0.58**
	CD (P=0.01)	**1.84**	**1.64**	**1.72**	**1.78**

* Mean of three replications, Dia.: Diameter
Figures in parentheses are arcsine transformed values

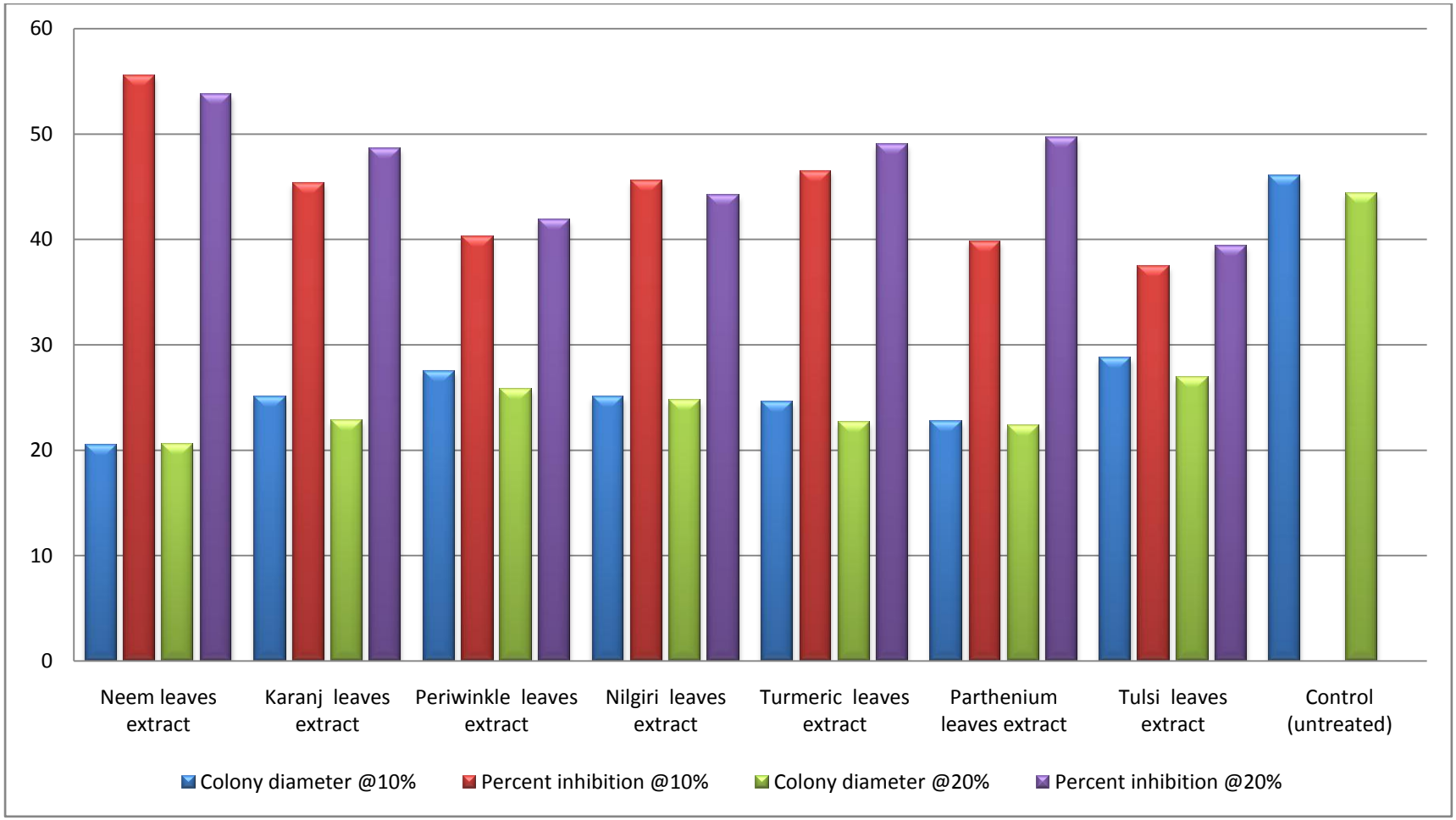
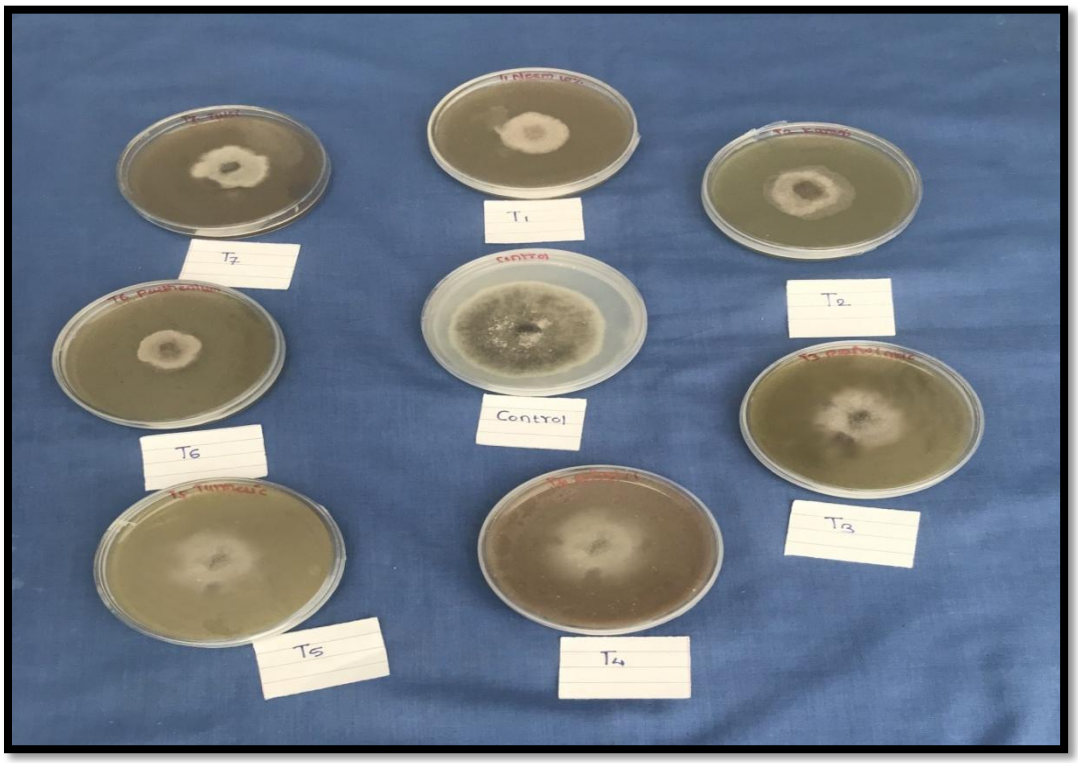
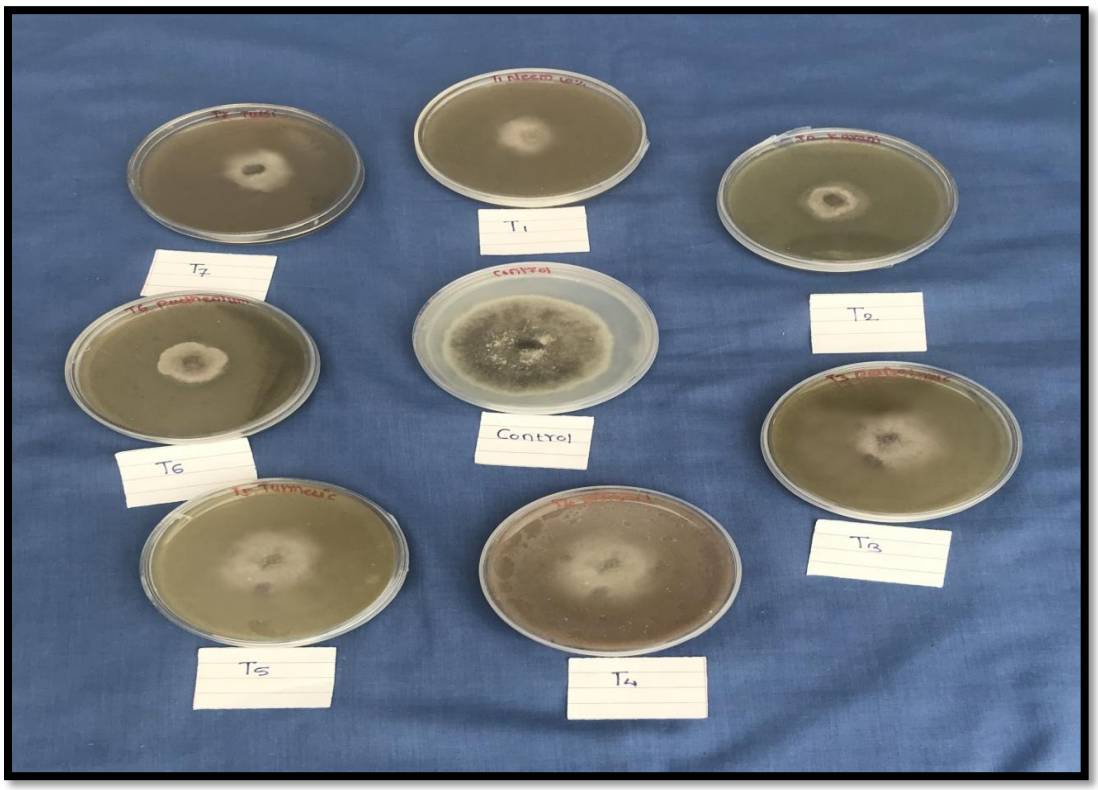


Fig.4.4: *In vitro* efficacy of Different Phytoextracts against *Alternaria alternata.*



Phytoextract Treatment @ 10%



Phytoextract Treatment @ 20%

Plate 4.8. *In vitro* efficacy of different phytoextracts against *Alternaria alternata.*

4.9. *In vitro* efficacy of different essential oils against *Alternaria alternata.*

Seven different essential oils were evaluated at 2000 and 2500 ppm concentration against *Alternaria alternata* by applying poisoned food technique and the results so obtained are presented in Table 4.8 and Fig 4.5. The results revealed that all the tested essential oils exhibited a wide range of radial mycelial growth of *Alternaria alternata* over untreated control (53.43 mm) and was found to be decreased with the increased concentration of plant extracts tested (Plate 4.9).

At 2000 ppm concentration of essential oils, the mycelial growth of *A. alternata* was observed in the range of 00.00 mm to 53.43 mm. There were no growth of *Alternaria alternata* in the treatments of Citronella oil, Clove oil and Rose oil (00.00 mm). The next best treatments followed by this were Ginger oil (20.20 mm), Eucalyptus oil (23.00 mm) and Lemon oil (23.51 mm). Amongst all essential oils, Cardamom oil was found comparatively less effective with having maximum growth of pathogen i.e.32.43 mm.

At 2500 ppm concentration of essential oils, the mycelial growth of *A. alternata* was observed in the range of 00.00 mm to 44.67 mm. The same pattern of growth was observed i.e no growth of *Alternaria alternata* in the treatments Citronella oil, Clove oil and Rose oil (00.00 mm). The next best treatments followed by this were of Lemon oil (12.88 mm), Eucalyptus oil (22.76 mm) and Ginger oil (23.66 mm). Amongst all essential oils, Cardamom oil was found comparatively less effective with maximum growth of pathogen i.e.25.44 mm.

Among the essential oils tested at 2000 ppm, cent per cent inhibition of the test pathogen was observed in the treatment of Citronella, Clove and Rose oil, which was followed by Ginger oil (62.19%), Eucalyptus oil (56.95%) and Lemon oil (55.83%). Amongst all essential oils, Cardamom oil was found comparatively less effective with minimum mycelial inhibition of pathogen i.e. 39.30 per cent.

Among the essential oils tested at 2000 ppm, Citronella, Rose and Clove oil were found most significant with highest mycelial growth inhibition of the test pathogen i.e. 100 per cent, which was followed by Lemon oil (71.16%), Eucalyptus oil (49.04%) and Ginger oil (47.03%). Amongst all essential oils, Cardamom oil was found comparatively less effective with minimum mycelial inhibition of pathogen i.e. 43.03 per cent over untreated control.

Similar effects of the different essential oils against *Alternaria alternata* infecting Pomegranate and many other crops were reported earlier by several workers. Gadhi *et al.* (2020) tested some essential oils such as Castor, Jasmine, Clove, Sesame, Neem, Coconut, Henna, Black seed, and Mint oil at different doses of 1, 2, 4, and 6 per cent against *A. alternata*. The results showed that maximum colony growth inhibition of *A. alternata* was recorded by Sesame (80.00%), followed by Coconut (77.04%), Henna (72.59%), Mint (66.07%), Black seed (71.85%), Jasmine (64.07%), Clove (70.74%), Neem (73.33%) and Castor (58.89%), whereas minimum inhibition (1.00%) was recorded in control.

4.10 *In vitro* bioefficacy of Ecolaid microbicide against *A.alternata.*

Four different Ecolaid microbicides were evaluated at different concentrations against *Alternaria alternata* by applying poisoned food technique and results so obtained are presented in Table 4.9 and Fig-4.6. The results revealed that all tested Ecolaid microbicide exhibited a wide range of radial mycelial growth of *Alternaria alternata* over untreated control (25.15 mm) and was found to be decreased with increased concentration of treatment (Plate 4.10).

At 250 ppm, 500 ppm concentration of Ecolaid microbicide and dormulin combi and dormulin (fruiting and flowering) at 1 gm per 100 ml concentration of microbocides, the mycelial growth of *Alternaria alternata* was observed in the range of 00.00 mm to 25.15 mm. The least growth of *Alternaria alternata* was observed in the treatments of Ecolaid microbicide and Ecolaid microbicide V2 (00.00 mm). The next best treatment followed by this was Dormulin combi (15.24 mm) and Dormulin (Fruiting and flowering) (18.77 mm). Amongst all Ecolaid microbicides Dormulin (Fruiting and flowering) was found comparatively less effective with maximum mycelial growth of *Alternaria alternata* i.e.18.77 mm.

Among all the Microbicide tested Ecolaid microbicide and Ecolaid microbicide V2 at 250 ppm and 500 ppm concentration were found most significant with highest mycelial growth inhibition (100 %) of the test pathogen. They were followed by Dormulin combi (39.40%). Amongst these microbicide, Dormulin (fruiting and flowering) was found less effective with 25.36 per cent inhibition of test pathogen as compared to other treatments.

Table: 4.8. *In vitro* efficacy of different essential oils against *Alternaria alternata.*

Sr. No.	Treatments	Colony dia.*(mm)		Percent inhibition	
		2000 ppm	**2500 ppm**	**2000 ppm**	**2500 ppm**
T1	Lemon oil	23.51	12.88	55.83 (48.34)	71.16 (57.51)
T2	Eucalyptus oil	23.00	22.76	56.95 (48.99)	49.04 (44.44)
T3	Rose oil	00	00	100 (90)	100 (90)
T4	Ginger oil	20.20	23.66	62.19 (52.05)	47.03 (43.29)
T5	Clove oil	00	00	100 (90)	100 (90)
T6	Citronella oil	00	00	100 (90)	100 (90)
T7	Cardamom oil	32.43	25.44	39.30 (38.82)	43.03 (40.99)
T8	Control (untreated)	53.43	44.67	00 (00)	00 (00)
---	**SE ±**	**0.43**	**0.38**	**0.43**	**0.51**
---	**CD (P=0.01)**	**1.77**	**1.58**	**1.78**	**2.13**

* Mean of three replications, Dia.: Diameter
Figures in parentheses are arcsine transformed values.

Table 4.9 *In vitro* bioefficacy of Ecolaid microbicide against *A.alternata.*

Tr. No.	Treatments	Rate of application per 100 ml	Colony Dia. of test pathogen * (mm)	% Inhibition
T1	Ecolaid microbicide	250 ppm	00	100 (90)
T2	Ecolaid microbicide	500 ppm	00	100 (90)
T3	Ecolaid microbicide V2	250 ppm	00	100 (90)
T4	Ecolaid microbicide V2	500 ppm	00	100 (90)
T5	Dormulin Combi	1 gm	15.24	39.40 (38.88)
T6	Dormulin (Fruiting and flowering)	1 gm	18.77	25.36 (30.23)
T7	Control (untreated)		25.15	00.00 (00)
	SE±		**0.20**	**0.44**
	CD (P=0.01)		**0.85**	**1.84**

* Mean of three replications, Dia.: Diameter
Figures in parentheses are arcsine transformed values.

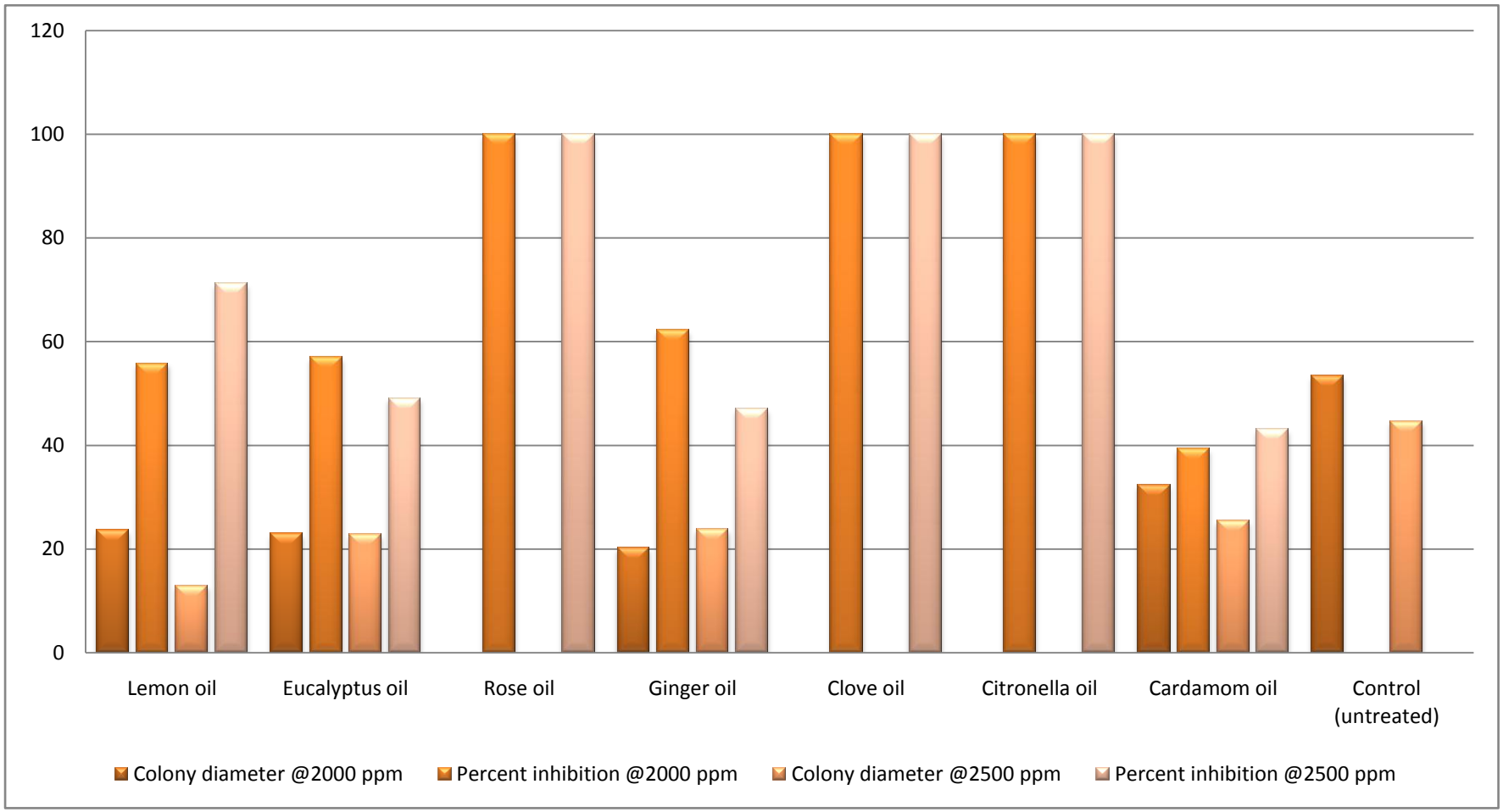
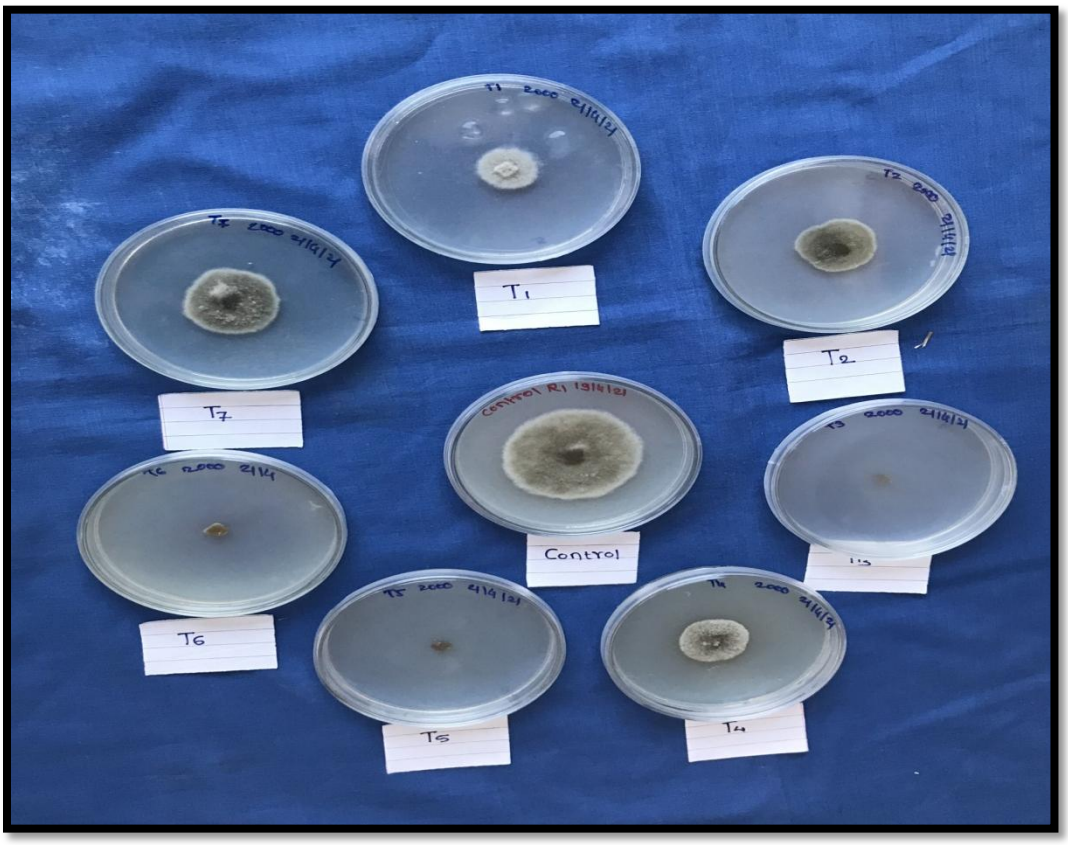
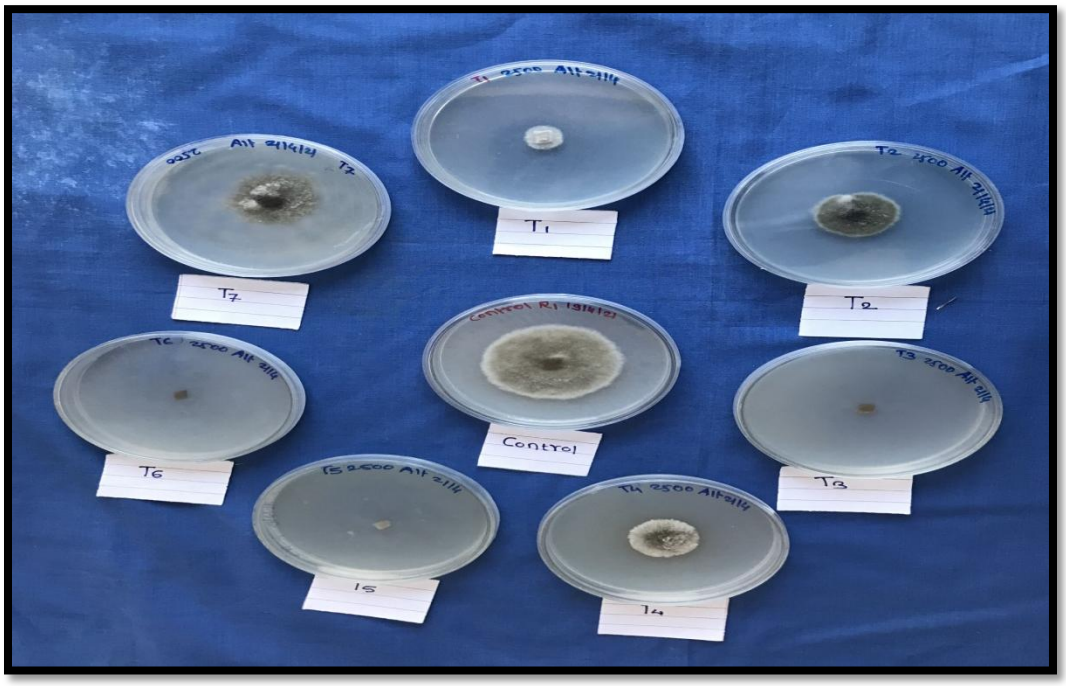


Fig.4.5: In vitro efficacy of Different Essential oils against *Alternaria alternata*.



Essential oil at 2000 ppm



Essential oil at 2500 ppm

Plate 4.9. *In vitro* efficacy of different essential oils against *Alternaria alternata.*

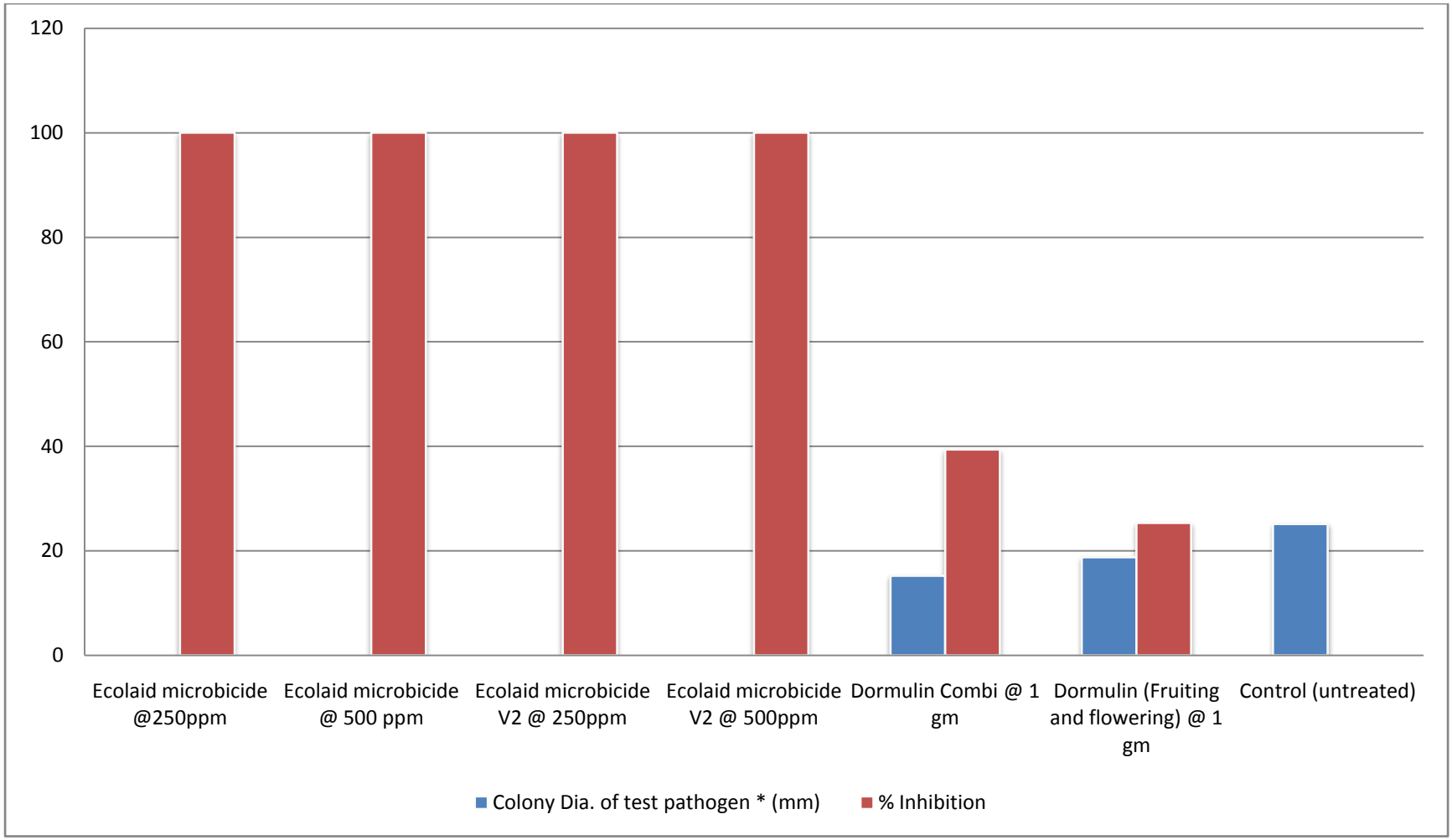


Fig.4.6: In vitro bioefficacy of Ecolaid microbicide against *Alternaria alternata.*

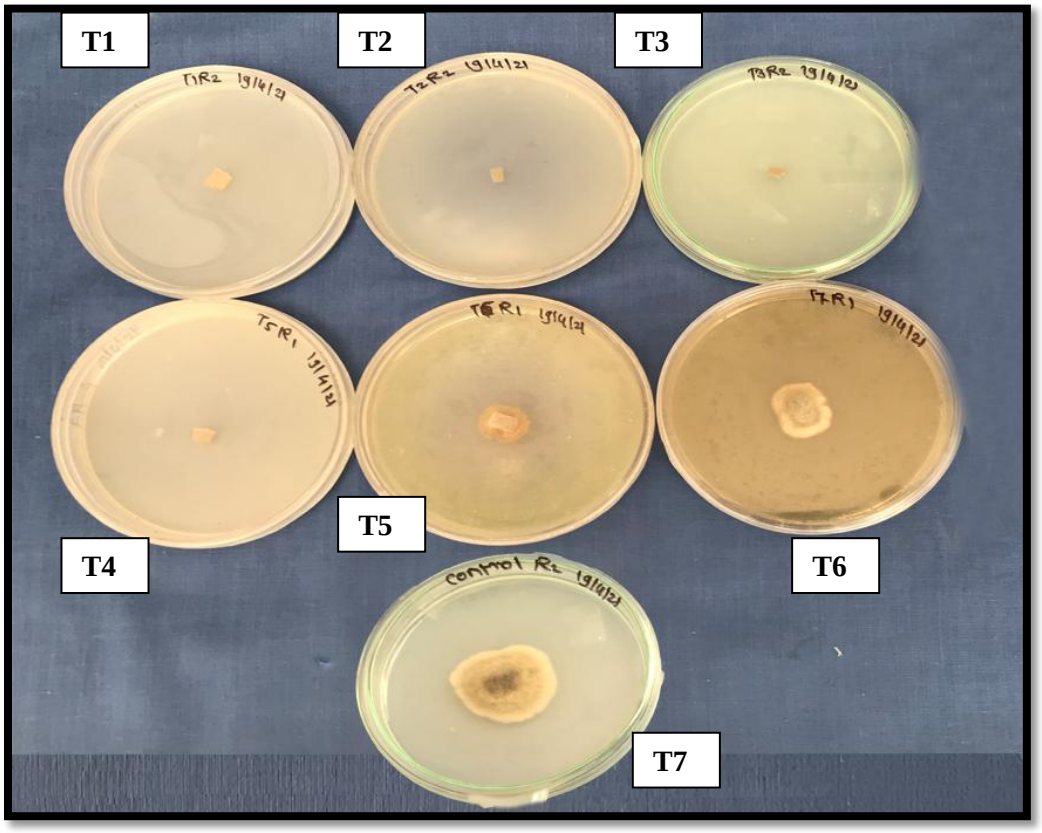


Plate 4.10. *In vitro* bioefficacy of different ecolaid microbicides against *Alternaria alternata.*

CHAPTER - V

SUMMARY AND CONCLUSIONS

CHAPTER-V

SUMMARY AND CONCLUSIONS

Pomegranate is a finest gift from Iran to central and northern India which is considered as super food. Now a days the export quality of pomegranate is decreasing due to many post harvest diseases as well as the loss in storage is also worsening due to fruit rot and heart rot caused by several post harvest pathogens among which *Colletotrichum gleosporioides* and *Alternaria alternata* are being major one with losses accounting upto 15-80 per cent (Kumar *et al.,* 2017). So, meticulous investigation is carried out on "Studies on heart rot of Pomegranate caused by *Alternaria alternata*" during 2020-21 at Department of Plant Pathology, College of Agriculture, Parbhani and NRC on Pomegranate, Solapur. The results obtained were summarized here after.

Alternaria spots were observed on leaves and fruit surface of Pomegranate. On leaves, symptoms showed round black regular to irregular spots with concentric rings, whereas, on fruits, the spots showed erupted, minute, unevenly distributed spots with green/yellow/red surroundings. Heart rot symptoms showed yellowing of rind without any visual symptoms on peel surface. Internal rotting was noticed with blackening of arils and membrane separating each compartment. The pathogenicity was proved for the isolated pathogen. Isolated pathogen has been proved as *Alternaria alternata* through the cultural, morphological and molecular studies. Pure culture of this pathogen was obtained by hyphal tip isolation.

In the cultural and morphological studies of *Alternaria alternata*, it was observed that, Oat meal agar media was found most suitable for growth of *Alternaria alternata* followed by Malt extract agar media. Amongst all the tested media, the least growth of *Alternaria alternata* was recorded on Richard's agar media.

All the seven bioagents evaluated *in vitro* were found effective against *Alternaria alternata.* However, *T. asperellum* was promising which inhibited 81.48 per cent mycelial growth of *Alternaria alternata.* Other *Trichoderma* species were also found effective in controlling the pathogen *in vitro*. The least mycelial inhibition of *Alternaria alternata* (44.36 %) was recorded in the treatment of *Bacillus subtilis*.

In vitro efficacy of phytoextracts against *Alternaria alternata* were tested @10 and 20 per cent. Amongst the tested phytoextracts, Neem leaf extract at 10 and 20

per cent was found most significant with highest mycelial growth inhibition i.e 55.54 per cent and 53.74 per cent, respectively, while Tulsi leaf extract at 10 and 20 per cent was found least effective against *Alternaria alternata* with 37.74 and 39.35 per cent mycelial growth inhibition, respectively.

In vitro efficacy of essential oils against *Alternaria alternata* were tested @ 2000 and 2500 ppm. Among them Citronella, Rose and clove oils were found most effective with highest mycelial growth inhibition of 100 per cent at both concentrations, whereas, Cardamom oil at 2000 and 2500 ppm were found least effective with 39.30 and 43.03 per cent growth inhibition of *Alternaria alternata*, respectively.

Besides this, different types of Ecolaid microbicides were also tested against *Alternaria alternata*. Among these all Ecolaid microbicides, the Ecolaid microbicides evaluated at 250 and 500 ppm were found most effective with cent per cent mycelial growth inhibition of test pathogen, whereas, Dormulin (fruiting and flowering) was least effective with 25.36 per cent mycelial growth inhibition of test pathogen.

CONCLUSIONS

Thus, from the results obtained on various aspects during present studies on "Studies on heart rot of pomegranate caused by *Alternaria alternata* (Keissl)." following conclusions are being drawn,

- Fruit rot and Heart rot caused by *Alternaria alternata* are important and destructive post harvest diseases which leads to reduction in export quality, heavy loss in plant stand per unit area and ultimately the yield.
- The pathogen *Alternaria alternata* survives best on Oat meal Agar medium and shows excellent sporulation. However, Malt extract agar and Czapek's Dox agar are most suitable for the pathogen.
- *Trichoderma asperellum* can be used as a most promising biocontrol agent against *Alternaria alternata.*
- The essential oil *viz.* Rose oil, Clove oil and Citronella oil has antifungal activities and can be used against *Alternaria alternata.*
- Neem leaf extract are most promising phytoextract and can be used to control *Alternaria alternata.*

- Ecolaid microbicide are emerging microbicide and these can be used even at low concentration that is 250 ppm and 500 ppm against *Alternaria alternata.*

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APPENDICES

APPENDIX - I

Composition of various culture media used for *in vitro* studies

1. Potato dextrose agar

In most of the experimental studies the Potato dextrose agar (PDA) was used. The composition of the PDA is as follows.

1. Potato (peeled) : 200.00 g.
2. Dextrose ($C_6H_{12}O_6$) : 20.00 g.
3. Agar-agar : 20.00 g.
4. Distilled water (Volume to make up) : 1000.00 ml

The extract was obtained by filtering through muslin cloth after 200 grams of peeled potatoes were chopped into minute pieces and cooked in distilled water. Dextrose and agar (20 gm each) were dissolved in the potato extract, and the final volume was brought up to 1000 ml with distilled water, sterilised, and stored as described earlier.

2. Malt extract agar

1. Malt extract :3 gm
2. Yeast extract:2 gm
3. KH_2PO_4:0.5 gm
4. $MgSO_4.7H_2O$: 0.5 gm
5. Agar: 20 gm
6. Distilled water: 1000 ml

In 400 ml of distilled water, mix malt extract powder thoroughly. Agar-agar was boiled separately in 400 ml of distilled water. The malt extract combination was combined with molten agar to make a volume of 1000 ml.

3. Saboraud's agar

1. Dextrose:20 gm
2. Peptone: 10gm
3. Agar :20 gm
4. Distilled water: 1000 ml

In 400 ml of distilled water, the above mentioned materials were thoroughly mixed. Agar-agar was boiled separately in 500 ml distilled water. The melted agar was fully combined with the solution. The volume was adjusted to 1000 ml. with distilled water.

4. Richard`s agar

1. Potassium nitrate (KNO_3) : 10.00 g
2. Potassium dihydrogen phosphate (KH_2PO_4) : 5.00 g
3. Magnesium sulphate ($MgSO_4$ $7H_2O$) : 2.50 g
4. Ferric chloride ($Fecl_3$ $6H_2O$) : 0.02 g
5. Sucrose ($C_{12}H_{22}O_{11}$) : 50.00 g
6. Agar – agar : 15.00 g
7. Distilled water : 1000.00 ml

Before sterilisation, all of the materials were dissolved one by one in 400 ml distilled water, and the agar was dissolved separately in 500 ml distilled water and mixed with the above solution, bringing the total volume to one litre.

5.Czapeck’s Dox agar

1. Sucrose ($C_{12}H_{12}O_{11}$) : 30.00g
2. Sodium nitrate ($NaNO_3$) : 2.00 g
3. Magnesium sulphate ($MgSO_4$. $2H_2O$) : 1.00 g
4. Magnesium sulphate ($MgSO_4$. $2H_2O$) : 0.50 g
5. Potassium chloride (KCl) : 0.50 g
6. Ferric chloride ($FeCl_3$. $6H_2O$) : 0.01 g
7. Agar-agar : 20.00 g
8. Distilled water : 1000.00 ml

In 500 ml distilled water, agar-agar was melted. The other materials were well mixed in another beaker containing 500 ml distilled water both mixtures were mixed thoroughly, and the volume was adjusted to 1000 ml and sterilised.

6. Oat Meal agar

1. Oat Meal: 20 gm

2. Agar : 20 gm
3. Distilled water: 1000 ml

Oat flakes were cooked for 30 minutes in 400 ml of distilled water, and the extract was filtered through muslin cloth. Agar-agar was cooked separately in 400 ml of distilled water. The obtained extract was thoroughly mixed into the molten agar, and the volume was adjusted to 1000 ml by adding distilled water.

7. V8 Juice agar

1. V8 juice :200 ml
2. $CaCO_3$
3. Agar : 15 gm
4. Distilled water: 800 ml

In 300 ml of distilled water, the above mentioned materials were well mixed. Agar-agar was cooked separately in 500 ml distilled water. The melted agar was fully combined with the solution. The total volume was 1000 ml. Sterilized medium for 15 minutes at 121.1 degrees Celsius and 15 pounds of pressure.

APPENDIX - II

Reagents used in molecular analysis

Sr. no.	Chemicals	Manufacturer
1	Uniflex DNA Isolation Kit	Himedia
2	Taq DNA polymerase	Himedia
3	10X Taq buffer E	Himedia
4	dATP	Himedia
5	dCTP	Himedia
6	dGTP	Himedia
7	dTTP	Himedia
8	Nuclease free water	Himedia
9	6X loading dye	Himedia
10	SYBR DNA staining dye	Invitrogen
11	100bp Ladder	Himedia
12	AxyPrep PCR Clean up kit	Axygen
14	50X TAE buffer	Himedia
15	Agarose	Himedia

APPENDIX - III

Equipments used in molecular analysis

Sr. no.	Equipment	Manufacturer
1.	PCR threrm ocycler	Himedia
2.	Water bath	Meta Lab
3.	Horizontal Electrophoresis	Himedia
4.	UV-visible Spectrophotometer	Shimadzu
5.	Weighing Balance (0.01g - 300g)	Citizen
6.	Micro Centrifuge (RM-12C)	Labquest
7.	Micropipettes	Himedia
8.	Deep Freezer	Blue star

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