DEVELOPMENT OF BIO-FORMULATION BASED ON BOTANICALS AND YEASTS AGAINST *Alternaria* spp. CAUSING LEAF SPOTS IN CAULIFLOWER

Thesis

by

SONALI PARWAN
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Dr. Harender Raj Gautam
Professor & Head

Department of Plant Pathology
College of Horticulture
Dr. Yashwant Singh Parmar University of Horticulture & Forestry
(Nauni) Solan (HP) – 173 230 India

CERTIFICATE-I

This is to certify that the thesis titled, **“Development of bio-formulation based on botanicals and yeasts against *Alternaria* spp. causing leaf spots in cauliflower”** submitted in partial fulfillment of the requirements for the award of the degree of **MASTER OF SCIENCE (AGRICULTURE) PLANT PATHOLOGY** in the discipline of **PLANT PROTECTION** to Dr. Yashwant Singh Parmar University of Horticulture and Forestry, (Nauni) Solan (HP) – 173 230 is a bonafide research work carried out by **Ms. Sonali Parwan (H-2019-73-M)** daughter of Shri Parvesh Parwan under my supervision and that no part of this thesis has been submitted for any other degree or diploma.

The assistance and help received during the course of this investigation have been fully acknowledged.

Place: Nauni, Solan
Dated:

Dr. Harender Raj Gautam
Chairman
Advisory Committee

CERTIFICATE-II

This is to certify that the thesis titled **"Development of bio-formulation based on botanicals and yeasts against *Alternaria* spp. causing leaf spots in cauliflower"**, submitted by **Ms. Sonali Parwan (H-2019-73-M)** daughter of Shri Parvesh Parwan to the Dr. Yashwant Singh Parmar University of Horticulture and Forestry, (Nauni) Solan (HP) – 173 230 India in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (AGRICULTURE) PLANT PATHOLOGY** in the discipline of **PLANT PROTECTION** has been approved by the Advisory Committee after an oral examination of the student in collaboration with the Internal Examiner.

14.09.2021

Dr. Harender Raj Gautam
Professor & Head
(Major Advisor)

Dr. Bharti Kashyap
Associate Professor
(Internal Examiner)

14/09/2021

Dr. S.C. Verma
Principal Scientist
(Dean's Nominee)

Members of Advisory Committee

1. **Dr. H. R. Sharma**
(Professor and Head)
Department of Vegetable Science

2. **Dr. Narender K. Bharat**
(Principal Scientist)
Department of Seed Science and Technology
14.9.21

Professor and Head
Department of Plant Pathology

Dean
College of Horticulture
Dr. Yashwant Singh Parmar University of Horticulture and Forestry
Nauni, Solan (HP) – 173 230

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"Where you begin doesn't matter. Your willingness to start is what counts"

(Rhonda Britten)

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I solely owe the responsibility for all the errors, shortcomings, omissions and limitations in this work.

Place: Nauni, Solan
Date: **(Sonali Parwan)**

CONTENTS

LIST OF ABBREVIATIONS

%	:	Per cent
>	:	Greater than
/	:	Per
@	:	At the rate
±	:	Plus or minus
µl	:	Microlitre
µm	:	Micrometer
BOD	:	Biological Oxygen Demand
cm	:	Centimeter
CRD	:	Completely Randomized Design
cv.	:	Cultivars
dia.	:	Diameter
et al.	:	etalia (Co – workers)
etc.	:	etcetera (and rest)
g	:	Grams
h	:	Hours
pv.	:	Pathovar
ha	:	Hectare
i.e.	:	Id Est (that is)
kg	:	Kilograms
m	:	Meter
mg	:	Milligram
f.sp.	:	Forma specialis
ml	:	Milliliter
mm	:	Millimeter
MSS	:	Mean sum of square
no.	:	Number
^{o}C	:	Degree centigrade

ºF	:	Degree Fahrenheit
rpm	:	Revolutions mer minute
ppm	:	Parts per million
Q	:	quintal
RBD	:	Randomized Block Design
H.P	:	Himachal Pradesh
psi	:	Pounds per square inch
DAT	:	Days After Transplanting
sp.	:	Species
SS	:	Sum of square
viz.	:	Vide licet (namely)
EC	:	Emulsifiable Concentrate
WP	:	Wettable Powder

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Chapter-1

INTRODUCTION

Cauliflower *(Brassica oleracea* var. *botrytis)* is one of the most important vegetable crops belonging to family Brassicaceae and it has originated in the island of Cyprus. This is usually a cool season vegetable where most of the varieties grow best at temperatures between 60ºF and 68ºF. In India it is cultivated in 458 thousand ha area with production of 8840 thousand metric tonnes (NHB, 2020). Cauliflower is cultivated in almost all the states in India, but states like Bihar followed by Uttar Pradesh, Orissa, Assam, Madhya Pradesh, Gujarat, Haryana and Himachal Pradesh are the leading States in production. In Himachal Pradesh, it is cultivated in 5310 ha area with production of 124330 metric tonnes (Anonymous, 2019).

Like other crops, there are several factors which affect the production of the cauliflower crop and among these diseases caused by fungal, bacterial and viral pathogens is one of the major concerns. Diseases which cause serious economic losses include black rot (*Xanthomonas campestris* pv. *campestris)*, downy mildew (*Pernospora parasitica*), wire stem (*Rhizoctonia solani*), leaf spot/ blight (*Alternaria brassicicola, Alternaria brassicae* and *Alternaria raphani*), yellow or fusarium wilt (*Fusarium oxysporum* f. sp. *conglutinans*), black leg (*Phoma lingum*), club root (*Plasmodiophora brassicae*), sclerotinia/white rot (*Sclerotinia sclerotiaorum*), damping off (*Pythium debaryanum*), cauliflower mosaic (Cauliflower mosaic virus) and disorder like deficiency of molybednum (Whiptail disease) are important (Mal *et al.,* 2020).

Among fungal pathogens, *Alternaria* is the most dominant pathogen and different species of *Alternaria* like *A. brassicae*, *A. brassicicola*, *A. raphani*, *A. alternata* are major constraint in the production of cauliflower and other brassicacea crops (Kolte, 1985). Among these, *A. brassicae* is one of the important pathogen of cauliflower. In addition to causing leaf spot, *A. brassicae* also cause pre-mature defoliation and curd deterioration, which has been reported to reduction of as high as 55.9 per cent in the seed yield (Prasad and Vishunavat, 2006). Leaf spots caused by *Alternaria brassicae* have been reported to causes upto 35% damage in mustard (Kolte *et al*., 1987). Alternaria blight caused by *Alternaria brassicae* has been reported to cause 5-30 per cent losses in cauliflower (Pandey *et al.,* 2002). Tamayo *et al.*

(2001) reported upto 30% losses in cauliflower by *Alternaria brassicae* in Columbia and South America.

Symptoms of Alternaria leaf spot/ blight appear as small, dark circular areas of necrotic lesions on the leaves. As the disease progresses, circular lesions of size 1cm in diameter start forming which are gray, gray tan or nearly black in color. In humid weather, dark conidiophores are seen on the surface of the lesion in concentric rings. The disease is not only limited to leaves but also infects other parts of the plant including silliqua, seeds, stems. Disease is one of the constraints in seed production. Seedlings in the nursery are also infected with small dark spots on stem which leads to damping off or stunting of the plants. Alternaria blight is reported to cause blackening of fruit bearing branches, pre-mature defoliation of leaves, curd deterioration, pre-mature ripening of silliqua which has been found to cause as high as 80 per cent reduction in seed yield in *Brassica oleracea* (Maude *et al*., 1984).

Usually, chemical fungicides are used for the management of Alternaria leaf spots/ blight in cauliflower (Singh *et al.,* 1990). However, residues of pesticides have been reported in the edible parts of cauliflower which are bound to have adverse effects on the health of the consumers (Liu *et al.,* 1992; Logrieco *et al.,* 2009). Thus, there is a need for finding out the alternatives for the management of Alternaria leaf spots in cauliflower. There are many effective alternatives to chemical fungicides which either alone or in combination can effectively manage the diseases in different crops. Other methods, like use of bio-control agents, botanical pesticides, other plant products etc. have potential for the management of diseases (Jayapradha and Raja, 2016). In this study, efforts have been made to identify such locally available plants and yeasts which have anti-fungal properties against *Alternaria brassicae* so that the effective botanicals and yeasts can be combined to make effective formulation(s).

Hence, keeping in view the importance of the crop and the damage caused by the pathogen, the present studies are being proposed to evaluate the alternatives to chemical fungicides with the following objectives:

Objectives

i. To isolate the pathogen associated with Alternaria leaf spots of cauliflower

ii. To isolate the yeasts from different sources

iii. To evaluate the plants and the yeasts with anti-fungal properties against *Alternaria brassicae/ A.brassicicola* causing Alternaria leaf spots in cauliflower

iv. To evaluate the efficacy of different bio-formulations based on effective anti-fungal plants and yeasts against *Alternaria brassicae/ A.brassicicola* causing Alternaria leaf spots in cauliflower under *in vitro* conditions

Chapter-2

REVIEW OF LITERATURE

Alternaria leaf spots caused by different species of Alternaria is a serious problem in cauliflower (*Brassica oleracea* var. *botrytis*) around the globe (Michereff *et al.,* 2012). This is also a serious disease in India and Himachal Pradesh (Singh *et al.,* 2014; Bharat and Dogra, 2011). The literature pertaining to the important aspects of Alternaria leaf spots disease in cauliflower and other related crops has been reviewed in the context of the present studies under the following headings:

2.1 Geographical distribution of the pathogen

2.2 Yield losses

2.3 Symptomatology of the Disease

2.4 Proving Pathogenicity

2.5 Morphology of the pathogen

2.6 Disease Management

- **2.6.1 Disease management through Yeasts**
- **2.6.2 Disease management through Botanicals**
- **2.6.3 Integrated disease management**

2.1 GEOGRAPHICAL DISTRIBUTION OF THE PATHOGEN

There are numerous reports on the occurrence of Alternaria leaf spots of cauliflower from different parts of the world and the disease is reported to be caused by two species of *Alternaria i.e. Alternaria brassicae* (Gray leaf spot) and *Alternaria brassicicola* (Dark leaf spot) on crucifer vegetables causing huge losses in yield (Hodgkin and Macdonald, 1986; Dillard *et al.,* 1998; Pattanamahakul and Strange, 1999), including India (Meena *et al.,* 2012b; Sharma *et al.,* 2013a).

Alternaria species are potential cosmopolitan fungi under the division of Ascomycota and can be found in soil, plant, food, feed and indoor air (Nayyar *et al.,* 2014). *Alternaria* as a genus is a serious pathogens in different crops. Nees first described the genus *Alternaria* in 1817, initially named *Alternaria tenuis,* which was later renamed as *Alternaria alternata.* Berkeley (1836) noticed symptoms similar to the diseases caused by this pathogen in the family Brassicaceae but initially identified this fungus as *Macrosporium brassicae* Berk., which was later renamed as *Alternaria brassicae* (Berk.) Sacc by Saccardo (1886). Specifically, *Alternaria brassicicola* was first recorded in 1924 from Holland on cabbage (Bolle, 1924). Mason (1928) first reported *Alternaria* from Pusa (Bihar) on a preserved herbarium material of sarson (*Brassica* sp.) in India. Petrie (1974) reported from western Canada that more than 50 per cent seed samples of different cultivars of cauliflower carried *Alternaria brassicicola* infection. *Alternaria* is greatly influenced by weather and wet season and in areas with relatively high rainfall had higher incidence of the disease (Humpherson-Jones and Phelps, 1989).

Alternaria species are important fungal pathogens of cruciferous crops including economically important *Brassica* species which causes spots on different aerial plant parts (Cramer and Lawrence, 2004). Gaetan and Madia (2005) reported gray leaf spot disease of canola caused by *Alternaria brassicae* (Berk.) Sacc. from Argentina. Alternaria Leaf Spot caused by *Alternaria brassicae* was first reported on *Crambe abyssinicia* from Australia (You *et al.,* 2007). *Alternaria* species are reported to cause black spot in different crucifer crops like oil seed rape, cabbage, cauliflower and mustard from countries like Italy (Tosi and Zezzerini, 1985); USA, UK and several other European countries (Gladders, 1987); Canada (Berkeramp and Kirkham, 1989; Conn and Tewari, 1990), Iran (Nourani *et al.,* 2008) and India (Meena *et al.,* 2010). In India, Chauhan *et al.* (2009) carried out a survey of diseases caused by genus *Alternaria* on plants of *Brassicaceae* family and reported different species of *Alternaria* namely *A. brassicicola, A. brassicae* and *A. raphani* on plants of Brassicae family.

2.2 YIELD LOSSES

Foliage and seed pods of crucifer crops are reported to be severely damaged affecting the seed germination due to Alternaria blight caused by *Alternaria brassicae* (Kolte 1985; Kolte and Awasthi, 1989; Tewari, 1991; Verma and Saharan, 1994) and *Alternaria brassicicola* (King, 1994; Jung *et al.,* 2002). *Alternaria brassicicola* is the dominant pathogen in cole crops, especially cauliflower and cabbage, and it causes more severe diseases than *Alternaria brassicae* (Maude and Humpherson-Jones, 1980; Humpherson-Jones, 1988; Maude *et al.,* 1984; Kubota *et al.,* 2006). In addition to damage in the standing crop, considerable damage to cabbage is reported due to Alternaria leaf spots in transit (Ellis, 2001).

Humpherson-Jones (1989) reported a total loss in seed yield up to 86% in Europe due to *Alternaria* species infecting *Brassica oleracea*. Losses up to 30% in crucifer crops due to Alternaria leaf spot have been reported from Columbia (Tamayo *et al.,* 2001). Yield losses due to Alternaria infection had been reported to be 32-57% in Nepal (Shrestha *et al.,* 2005; Saharan *et al.,* 2016). Alternaria leaf spots caused by seed borne pathogen *Alternaria brassicicola* is responsible for 9 to 28% loss in yield (Kedian and Saharan, 1983). *Alternaria* species are reported to cause reduction in seed yield may be high as 80% in mustard and other cruciferous vegetable crops (Smith and Taylor, 1992). *Alternaria brassicicola* is an opportunistic pathogen on numerous hosts causing at least 20% of agricultural spoilage and in case of severity of the disease, the losses may reach up to 80% of the yield. (Nowicki *et al.,* 2012).

In India, Kolte (1985) has reported a yield loss up to 47% in Indian mustard infected with leaf blight due to *Alternaria brassicae* and *Alternaria brassicicola.* Huq *et al.* (1999) reported yield losses in many other crops due to different species of *Alternaria*. Yield losses of cabbage seed due to Alternaria blight (*A. brassicicola*) were recorded to be 59% (Hossain and Mian, 2005a). Hossain and Hossain (2010) reported that Alternaria blight may cause about 47.8% reduction in yield of cauliflower seed. Alternaria blight disease caused by *Alternaria brassicae* has been reported as one of the most important disease of rapeseed mustard causing severe yield losses (Meena *et al.,* 2010). In other studies, yield losses due to Alternaria infection had been reported to be 10-70% in India (Singh *et al.,* 2017; Choudhary *et al.,* 2018).

2.3 SYMPTOMATOLOGY OF THE DISEASE

Alternaria blight is caused by *Alternaria brassicae* (Berk.) Sacc. which is an important fungal problem that can severely affect the foliage and seed germination in crucifers (Weiss, 1983; Kolte, 1985; Tewari, 1991; Verma and Saharan, 1994). *Alternaria brassicicola* and *Alternaria brassicae* caused infection either singly or collectively and blacken the fruit-bearing branches and seed pods resulting in yield loss due to pre-mature pod ripening and shedding of seed (Maude and Humpherson-Jones, 1980). Spots produced by *A. brassicae* appear to be usually grey in color when compared with black sooty velvety spots produced by *A. brassicicola.*

Spots caused by *A. brassicicola* on the leaves are generally similar to those caused by *A. brassicae* except that the lesions are gray-black in colour (Kolte and Awasthi, 1989;

Verma and Saharan, 1994). Spots when young are dark brown in colour and can vary in size from 1.1 to 3.0 mm. Colour of the spots then changes to black, brown or tan and size varies from 5 to 7.5 cm when older (MacKinnon *et al.,* 1999; Singh *et al.,* 2012). Yellow halos may or may not surround leaf lesions. Larger spots may have a dark green-black coloration of fuzzy growth in the spots, usually concentrated in the center (Neergaard, 1945).

Verma and Saharan (1994) have also given a detail account of symptomatology of *Alternaria* leaf spots on plant hosts belonging to brassicae. They observed that in nursery beds, minute dark spots appeared on the cotyledons, stem of the seedlings, which led to a severe damping off and stunting of the young plants. Symptoms appeared on leaves as small dark brown to black spots, which enlarged to form concentric rings. Each spot was found to be surrounded by yellow-halo of chlorotic tissues. Older lesions are circular and frequently zonated, with a papery thin core covered with a mat of dark olive brown coloured spores in humid weather. In several cases, small dark coloured spots were reported on leaves and tender twigs (Valkonen and Kaponen, 1990).

Alternaria species also reported to cause black spot disease leading to damping off of seedlings; also cause symptoms like spots on the leaves in cabbages, back leg of heads of cabbages and spotting/ browning of cauliflower curds and broccoli florets (Nowicki *et al.,* 2012). *Alternaria brassicicola* spots were found darker and less regular in shape compared with those of *Alternaria brassicae.* The fungus can survive on susceptible weeds or perennial crops (Rangel, 1945; Chupp and Sherf, 1960; Maude and Humpherson-Jones, 1980).

Alternaria blight causes irregular brown to dark brown coloured spots, surrounded by concentric rings, give a "target board" effect appear on leaves. These circular spots coalesce and form large patches (Sohaib *et al.,* 2018). In other description of the symptoms, *Alternaria brassicicola* is reported to causes leaf spot, which starts as small dark spots that quickly spread to form circular lesions up-to 1cm in diameter (Pun *et al.,* 2020). A yellow halo surrounded the lesions, with alternating light and dark concentric circles giving the appearance of target spots. When compared to *Alternaria brassicicola*, the large-spore type *Alternaria brassicae* causes a lot of the common symptoms and tends to remain larger and lighter in colour.

2.4 PROVING PATHOGENICITY

Different researchers have reported different procedures for inoculation of the plants for proving pathogenicity of *Alternaria* species in brassicacea plants. Conn and Tiwari (1989) observed the appearance of initial symptoms of *Alternaria brassicae* on Indian mustard (*Brassica juncea*) after 72 hours of inoculation with spore suspension 4×10^4 spores per ml on the fourth true leaves taken from 45 days old plant. Iacomi-Vasilescu *et al.* (2004) confirmed the pathogenicity of *Alternaria* spp. causing leaf spot in crucifers by spraying with a spore suspension of 10^5 spores per ml on 60-day-old plant with the help of an atomizer in the late evening. Doullah *et al.* (2006) tested the pathogenicity of *Alternaria brassicicola* on *Brassica rapa* by applying three different inoculation methods and reported that the detached leaf inoculation method was the most efficient, with symptoms appearing within 24 hours of inoculation of the fourth true leaves. Kashyap and Dhiman (2009) inoculated the leaves of potted cauliflower seedlings (45 days old) by spray inoculation with conidial suspension of 5×10^5 conidia per ml. The inoculated seedlings were covered with water sprayed perforated polythene bags for 48 h to ensure proper relative humidity and maintained on glasshouse (20-25ºC).

Deep and Sharma (2012) proved the pathogenicity of *Alternaria brassiciola* and *Alternaria brassicae* on cauliflower by making injury on the surface with a fine needle by pinprick method and symptoms were observed on leaves third day after inoculation in the inoculated leaves. In another study, 2 µl of 4×10^4 spores were inoculated with a fine needle on cauliflower leaves and were placed inside moist chambers in greenhouse conditions (Sharma *et al.,* 2013b). Siciliano *et al.* (2017) proved the pathogenicity of *Alternaria* spp. causing leaf spot on cabbage and cauliflower plants by raising them in pots for two months. The plants were then inoculated with conidial suspension at $1\text{-}5\times10^5$ conidial per ml using atomizer and symptoms were first observed on the old leaves.

2.5 MORPHOLOGY OF THE PATHOGEN

Most of the *Alternaria* spp. produces chains of conidia with transverse and longitudinal septa, with a tapering apical cell. Conidial size, presence and size of a beak, the pattern of catenation and longitudinal and transverse septation are key taxonomic features for this genus (Joly, 1964; Ellis, 1971 and 1976; Simmons, 2002). Ellis (1971) while conducting morphological studies on *Alternaria brassicicola* observed that the colonies of the pathogen as amphigenous, effused, dark olivaceous brown to dark blackish brown and velvety. Mycelium was reported to be immersed, branched and septate hyphae; hyaline at first, later brown or olivaceous brown, inter and intra-cellular, smooth, 1.5-7.5 µm in thickness. Humpherson-Jones and Phelps (1989) reported a temperature range of 18-24°C optimum for sporulation of *Alternaria brassicae* and 20-30°C for *Alternaria brassicicola.* In *Alternaria,* morphological characteristics of conidia and conidiophores and sometimes host plant association, provide the major taxonomic criteria for delimitation of fungal species (David, 1991).

The pathogen was reported to survive in seeds for several months at different temperatures and relative humidity (Abul-Fazal *et al.,* 1994; Kumar and Gupta, 1994) and spreads during the growing season by wind-blown or rain-splashed spores (MacKinnon *et al*., 1999). Khalid *et al.* (2004) has given a comprehensive and comparative account of morphological differentiation among different *Alternaria* species occurring on brassicaceous crops. Meena *et al.* (2010) gave detailed account of morphological characters of *A. brassicicola* where the mycelium was reported to be olive grey to greyish black with frequent septation at regular interval. Conidiophores were olivaceous, septate and branched measuring 35-45 µm in length and 11-16 µm in width while conidia were dark cylindrical to oblong, muriform without beak measuring 44-55 µm in length and 11-16 µm in width and produced in chains of 8-10 spores. The conidia were devoid of any prominent beak and found to have 5-8 transverse and 0-4 longitudinal septations. The fungus grows faster in media with high sporulation and appears as well developed black sooty colony with distinct zonation (Kolte, 1985).

2.6 DISEASE MANAGEMENT

Use of chemical fungicides is resulting in adverse effects on the crop produce, human health and environment. So there is need for exploring the possibilities for alternative methods of disease management. There are number of plants which possess anti-fungal properties against important plant pathogens. Eco-friendly methods, using bio control agents and many plant products to suppress plant disease, offers a powerful alternative tool to synthetic chemicals with similar targets. The rich diversity of microbial population and the availability of numerous medicinal plants provide a seemingly endless resource for this purpose. Many of the fungal pathogens causing foliar diseases in plant species viz. leaf spots, leaf blights and leaf blotch.

2.6.1 Disease management through yeasts

Yeasts are also potential bio control agents and are promising substitutes for chemical fungicides in the control of pre and post harvest decay owing to their widespread distribution, antagonistic ability, environment friendly nature and safety for humans (Xiaokang *et al.,*2020). The antagonistic yeasts are likely to function via multiple mechanisms, including competition for nutrients and space, mycoparasitism, induction of host resistance, production of volatile organic compounds (VOCs), and toxins (Freimoser *et al.,* 2019). Therefore, the competition for nutrients and space has been considered the primary mode by which antagonistic yeasts suppress postharvest fungal pathogens (Spadaro and Droby, 2016). Carbon, nitrogen, and iron ions are the main nutrients needed for the growth of microbes. Compared with carbohydrates, nitrogen is considered to be a key factor limiting the growth of postharvest fruit pathogens, because most fruits are rich in sugar but limited in nitrogen sources such as amino acids. Moreover, iron plays a crucial role in the growth and virulence of pathogens. Iron is a component of cytochromes, other heme proteins, and non-heme proteins; it is also a cofactor of various enzymes in fungal cells (Talibi *et al.,* 2014). Antagonistic activity of *Saccharomyces cerevisiae* is mostly due to the secretion of hydrolytic enzymes as well as organic volatile compounds in yeasts that have been characterized and studied with respect to their antifungal activity against *Colletotrichum acutatum* on citrus (Lopes *et al.,* 2015). Yeast can produce some plant growth-promoting enzymes, including indole-3-acetic acid (IAA), phosphate solubilization and reduce growth of plant-pathogen by releasing cell wall degrading enzymes (Fu *et al.,* 2016).

Sharma *et al.* (2009) demonstrated yeasts to antagonize common postharvest pathogens, including *Botrytis, Penicillium, Rhizopus, Colletotrichum, Monilinia, Aspergillus* and *Alternaria* under *in vitro* conditions. Rosa-Magri *et al.* (2011) reported the antagonism effect of the yeast *Torulaspora globosa* under *in vitro* conditions against the phytopathogenic mold *Colletotrichum graminicola*, the causal agent of anthracnose disease in maize. Abdel-Kadar *et al.* (2012) reported the effectiveness of *Saccharomyces cerevisiae, Trichoderma harzianum*, *Trichoderma viride, Bacillus subtilis* and *Pseudomonas fluorescens* as foliar sprays either alone or in combinations on vegetables such as Tomato, Cucumber, Cantaloupe and Pepper in open greenhouse conditions against early, late blight of tomato as well as powdery and downy mildew of Cucumber, Cantaloupe and Pepper. Potential of *Saccharomyces cerevisiae* as PGP (plant growth-promoting) and biocontrol agent has been highlighted for sustainable agriculture in harsh conditions (Amprayn *et al.,* 2012).

Among yeasts, *Saccharomyces cerevisiae* is the major and dominating microbe in the microbial community that can easily be found on the leaf, fruit and vegetables surfaces.

Parameswari *et al*. (2015) isolated yeasts from papaya and grape fruit pulps and the yeast were identified as *Saccharomyces* based on colony characteristics and biochemical tests. Yeast has been used for a long time as bio-control antagonist against different pathogen (Droby *et al.,* 2016).

Stress tolerance can also be provided by yeast (El-Zohri *et al.,* 2017). Kasfi *et al.* (2018) reported that among many species of yeasts isolated from the epiphytic flora associated with grape berries and leaves cv. 'Thompson seedless' from vineyards in Iran, two yeasts species including *Meyerozyma guilliermondii* and *Candida membranifaciens* had high antagonistic capability against the pathogen- *Botrytis cinerea*. Potential of *Saccharomyces cerevisiae* to inhibit the potential pathogen of different plants have been reported by many researchers (Mohammed *et al.,* 2008; Ibrahim and El-Fiki, 2018; Kamel *et al.,* 2016). Ferraz *et al.* (2019) has recently reported that yeasts can be used as potential biocontrol agents for phytopathogen causing cacao witches' broom disease caused by *Moniliophthora perniciosa*.

Five applications by yeasts i.e. *Saccharomyces cerevisiae*, *Pichia albicans*, *Candida sake* and commercial biocontrol agent Rhizo-N (*Bacillius subtilis*) as well as fungicide (Topsin-M 70%) significantly reduced powdery mildew (*Erysiphe betae*) disease incidence on sugar beet than untreated plants. Ziedan and Farrag (2011) reported that Topsin M-70% was the best treatment; it completely suppressed powdery mildew followed by *S. cerevisiae*, *C. sake* and *P. albicans* under field conditions. Foliar spray of *S. cerevisiae*, *P. albicans* and *C. sake* gave 76.15, 67.70 and 74.66 % against powdery mildew disease, while gave 26.06, 21.77 and 52.83% against Cercospora leaf spot disease, respectively.

Chen *et al.* (2018) evaluated 24 strains of yeast with antagonistic ability against gray mold and the antifungal activity of the volatile and diffusible metabolites was also evaluated. Putative mechanisms of action associated with the bio-control capacity of yeast strains against *B. cinerea* were studied through *in vitro* and *in vivo* assays. The volatile organic compounds produced by the *Galactomyces candidum* JYC1146 could be useful in the biological control of plant pathogens and therefore are potential alternative fungicides with low environmental impact.

2.6.2 Disease management through botanicals

Plants with antimicrobial activity offer great opportunity and potential alternative to chemical pesticides for their use in the management diseases in different crops. This will help to avoid the problem of soil and air pollution and health hazards associated with most of the toxic chemical pesticides to human beings and other living beings on the earth.

Extracts and oils from many plants have been reported to be effective against many important pathogens causing serious losses in our crops. There are many such reported about their effectiveness against different *Alternaria* species infecting our crops. Kumar *et al.* (1998) evaluated 25 botanicals against *Alternaria brassicae* under *in vitro* conditions and observed that the extract of *Solanum xanthocarpum* and *Datura innoxia* completely inhibited the spore germination at 10 per cent concentration. Sharma *et al.* (2007) reported that neem leaf extract showed high efficacy to inhibit the radial growth of *Alternaria solani* (43.3 and 26.7 % respectively at 0.1 and 0.01 %).

Sallam (2011) reported that out of the six plant extracts (*Ocimum basilicum, Azadirachta indica, Eucalyptus chamadulonsis, Datura stramonium, Nerium oleander* and *Allium sativum*) evaluated against *Alternaria solani* under *in vitro* conditions, leaf extracts of *Datura stramonium, Azadirachta indica* and *Allium sativum* at 5 per cent concentration caused 44.4, 44.3 and 42.2 per cent reduction of mycelia growth of *Alternaria solani,* respectively. Sasode *et al.* (2012) reported the effectiveness of Neem, Eucalyptus, Datura, Lantana, Tulsi and Pudina at 10 per cent concentration against *Alternaria brassicae* and *Alternaria brassicicola* infecting mustard under *in vitro* conditions. Extracts of *Canna indica, Ipomea palmata, Mentha piperita, Allium cepa, Allium sativum, Argemone mexicana, Datura stramonium* and *Clerodendron inerme* were reported to completely inhibit the spore germination of *Alternaria brassicae* infecting cauliflower (Sheikh and Agnihotri, 1972).

Cow urine contains phenolic acids viz., gallic caffeic, coumaric, cinnamic, and salicyclic acids which have antifungal characteristics (Singh *et al.,* 2015). However, it remains to be established whether the antifungal activity is a result of one or a combination of these phenolic acids. Addition of plant extracts in cow-urine may result in a synergistic antifungal effect against *Alternaria brassicicola*. Plants produce metabolites which have antifungal activities. The effectiveness of leaf extract of different botanicals in the present study can be corroborated to their fungitoxic effect due to presence of one or the other acid or organic molecules. Qurecetin and β-sitosterol, polyphenolic flavonoids, produced were known to have antibacterial and antifungal properties against many pathogenic fungi (Alzohairy, 2016).

Chamoli *et al.* (2020) evaluated that botanicals and bioagents against Alternaria leaf spot of cabbage caused by *Alternaria brassicicola* out of which maximum per cent mycelium growth inhibition was found in neem followed by garlic and tulsi.

Use of fungicides is the prevailing and commonly practiced method for the management of leaf spots caused by *Alternaria* species in Cole crops and other crops of Brassicaceae family. Repeated applications of fungicides are required to achieve the potential yields in infected crops. Captafol has been found more effective than Dithane M-45 for control of Alternaria blight of cauliflower to get maximum yield (Sinha and Prasad, 1989). Hussaini and Singh (1989) found Dithane M-45 (0.25%) more efficacious than Bordeaux mixture (0.4%) for the management of Alternaria blight of radish seed crop. Khan *et al.* (2007) evaluated the efficacy of different systemic and non-systemic fungicides against Alternaria blight of rapeseed mustard caused by *A. brassicae* both *in vitro* and *in vivo*. Foliar sprays of Mancozeb have also been reported effective in the management of early blight of tomato and Alternaria blight of oilseeds (Mondel *et al.,* 2008; Meena *et al.,* 2012a). Gondal *et al.* (2012) reported the efficacy of copper oxychloride and mancozeb in inhibiting the growth of the pathogen- *Alternaria solani*.

Thiram (80%) and Arasan (50%) proved to be the most successful fungicide which completely inhibits growth of Alternaria. Apart from this, Dithane M-45 was considerably superior fungicide against *Alternaria cucumerina* causing leaf blight of watermelon (Marrgain *et al.,* 2013). Panwar *et al.* (2013) reported complete inhibition of *A. alternata* in hexaconazole followed by mancozeb and least inhibition in carbendazim. Tu (2015) recorded complete inhibition of *A. brassicicola* by hexaconazole, mancozeb at 250, 500 and 1000ppm and by metalaxyl + mancozeb at 500ppm and 1000ppm and least inhibition at carbendazim.

Pun *et al.* (2020) showed the efficacy of different chemical fungicides against *Alternaria brassicicola* under *in vitro* using poisoned food technique. Hexaconazole proved to be the most effective fungicide showing complete inhibition (100%) followed by carbendazim + mancozeb (93.81%) and mancozeb (89.05%) while carbendazim (72.83%) followed by copper oxychloride (72.05%) were found to be least effective. In interaction effect, highest inhibition (i.e. 100%) of mycelial growth was recorded at all concentrations of hexaconazole, 1000ppm of mancozeb and metalaxyl + mancozeb, 500ppm of mancozeb which were significantly indifferent with 1000ppm carbendazim + mancozeb (99.71%), 500ppm of carbendazim + mancozeb (96.72%) and metalaxyl + mancozeb (95.74%) and 250ppm of mancozeb (98.83%).

2.6.3 Integrated disease management

Application of combination of different bio-control methods have been reported to be effective for the management of different diseases of different crops (Raja and Kurucheve, 1997; Himankshi, 2011). Buttermilk being a fermented product, rich in probiotics, has been reported to contribute towards plant growth promoting properties (Stiles and Holzapfel, 1997). Lactic acid is one of the major constituent of the buttermilk and it has been found to possess bio-control activity against phytopathogens (Trias *et al.,* 2008). Akhtar *et al.* (2006) evaluated plant extracts or their combination with cow urine and cow dung on conidial germination of *Biopolaris sorokinian* and reported that combination were found to be superior in comparison to the treatment of cow urine. Himankshi (2011) reported that mixture of bacterial and yeast isolates along with cow urine was found to be more effective in exhibiting antagonistic activity against all selected phytopathogens (*Alternaria brassicae*, *Colletotrichum truncatum*, *Sclerotium rolfsii*, *Fusarium oxysporum*, *Rhizoctonia solani*) and this may be due to the combined effect of antagonistic compounds secreted by probiotics and volatile and non-volatile compounds of cow urine which probably augmented this anti-microbial activity.

Combination of plant extracts and yeasts have also been reported to be effective due to the presence of phytohormones in the plant extracts. Salicylic acid (SA) is an important hormone in plants that is related to the induction of the plant response against pathogens (Romanazzi *et al.,* 2016). Qin *et al.* (2003) found that SA treatment increased the antagonism of *Rhodotorula glutinis* against *P. expansum* and *A. alternata* in sweet cherry fruits. SA at low concentrations increased the activities of defence-related enzymes but had little effect on the growth of the yeast and the two pathogens. This indicated that the biocontrol efficacy enhanced by SA may be related to the triggering of host resistance. Methyl jasmonate (MeJA) is another phytohormone that can induce host defence responses (Aloui *et al.,* 2015). MeJA has also been reported to improve the biocontrol effects of antagonistic yeasts (Guo *et al.,* 2014). Li *et al.* (2019) reported that cinnamic acid improved the bio-control efficacy of *Cryptococcus laurentii*, which indicates the potential of combined application of natural plant extracts with antagonistic yeasts for controlling postharvest pathogens.

Chapter-3

MATERIALS AND METHODS

The present studies entitled "**Development of bio-formulation based on botanicals and yeasts against *Alternaria* spp. causing leaf spots in cauliflower"** was carried out at experimental farm and laboratories of the Department of Plant Pathology, Dr. Yashwant Singh Parmar University of Horticulture and Forestry, Nauni, Solan, Himachal Pradesh during the year 2019-2021. **The methodologies adopted during the course of studies are elaborated with the following headings:**

3.1 Isolation and Identification

3.1.1 Isolation and Identification of the pathogen

3.1.2 Isolation and Identification of yeast isolates

3.2 Pathogenicity studies of the pathogen(s)

3.3 Morphological characters

3.4 Disease management

3.5 Microbial count of bio-formulations

3.6 Statistical analysis

3.1 ISOLATION AND IDENTIFICATION

3.1.1 Isolation and Identification of the Pathogen

Isolation

Infected leaves showing typical symptoms of the disease were used for the isolation of the test fungus by following standard procedure. Small bits of 1 to 3 mm size were taken from the junction of diseased and healthy portions with the help of sterilized blades. These bits were surface sterilized with sodium-hypochlorite (5%) for 10 to 20 seconds and washed thrice with sterilized distilled water. The bits were transferred on sterilized filter paper to remove excess moisture and subsequently transferred to Potato Dextrose Agar (PDA) slants under aseptic conditions. The inoculated slants were incubated for 4-5 days at 25±1ºC. Predominant colonies were re-isolated and purified by the hyphal tip method and maintained on PDA medium. Slants of pure culture were preserved in refrigerator at 4ºC and revived after 30 days to maintain the purity of test fungus (Pun *et al.,* 2020).

Identification of the pathogen

The morphological characters of the test fungus were studied on culture grown on PDA medium. Seven-day old culture was used for studying morphological characters of the test pathogen. Observations including colony color, hyphal color and septation, diameter, conidial shape, size (length and breadth) and the number of transverse and longitudinal septation was recorded using Olympus microscope and Promagnus software. These observations were compared with those of the standard measurements given by Ellis (1971) to identify the test fungus (*Alternaria* spp.).

Identification of the test fungus was confirmed with the help of Dr. PN Chowdhry, National Centre of Fungal Taxonomy, New Delhi.

3.1.2 Isolation and Identification of yeast isolates

Isolation

Yeasts were isolated from the phylloplane of the fruits/ raisins and chickpea using selective media i.e. Yeast Peptone Dextrose Agar (YPDA) media. A total of six isolates were isolated from different sources like fruits, raisins and chickpea. Two yeast isolates BK 1 and BK 4 were isolated from chickpea, two yeast isolates BK 5 and BK 6 from grapes, one yeast isolate BK 2 from apple pomace and one yeast isolate BK 7 from raisins. Five yeast isolates i.e. BK 1, BK 2, BK 4, BK 5, BK 6 were procured from Dr. Sanjeev Kumar Verma, VCSG Uttarakhand University of Horticulture and Forestry, Pauri, Uttarakhand and one yeast isolate i.e. BK 7 was isolated in the Department of Plant Pathology, Nauni, Solan.

Identification

Yeast isolates were maintained on Yeast Peptone Dextrose Agar slants and were used for further studies. The yeast isolates were studied for colony characteristics and cell shape (Parameswari *et al.*, 2015**)**. The observations of colony characters and cell shape were recorded by observing under Olympus microscope and Promagnus software.

Identification of the yeast isolates was confirmed with the help of Dr. PN Chowdhry, National Centre of Fungal Taxonomy, New Delhi.

3.2 PATHOGENICITY STUDIES

3.2.1 Preparation of inoculum

For inoculation, the suspension of spores (conidia) was made in sterile water and then spore suspension was sprayed on the plants. The spores were harvested by flooding a 10 day-old-colony on PDA medium with 10ml of sterilized distilled water and gently scraping the surface with a sterilized spatula to form spore suspension. The resulting spore suspension was then passed through a double layer of sterilized muslin cloth and finally concentration of 4×10^4 spores per ml was made with the help of haemocytometer (Deep and Sharma, 2012).

3.2.2 Inoculation Method

Pot culture

In order to prove the pathogenicity of the test fungus which was found to be associated with Alternaria leaf spots of cauliflower, plants of cultivar "Pusa Snowball K1" were raised in the earthen pots (10cm dia.) which were filled in with sterilized soil. After, attaining the age of 45 days, leaves of the plants were pin-pricked using a fine needle, and the spore suspension of the pathogen containing 4×10^4 spores/ml was sprayed using hand atomizer. Spore suspension was sprayed on both sides of leaves. Plants in the control treatment were sprayed with sterile distilled water. Observations were made regularly for the appearance and development of symptoms. After the appearance of disease symptoms, test fungus associated with diseased plants was re-isolated and compared with the original isolate (Gaikwad, 2013; Sharma *et al.,* 2013a).

Detached leaf technique

In order to prove the pathogenicity of the test fungal pathogen associated with Alternaria leaf spot of cauliflower, fully expanded leaves from 45 days old plant of cultivar "Pusa Snowball K1" were taken for inoculation. Healthy leaves were removed from the plants, washed with sterilized distilled water and placed in sterilized Petri plates. Detached leaves were moistened with the help of sterile distilled water and were pin-pricked at the two places on the abaxial leaf surface along the horizontal axis of the main vein. The incisions were inoculated with 20 µl of spore suspension (10^5 spores) were placed at the place of injury. While the sterilized distilled water was used for inoculation in control treatment. The leaves were placed in Petri plates (14 cm) lined on both sides with double-layered moist filter paper and incubated in Relative Humidity cum Temperature Control Cabinet adjusted at relative humidity of 95±5 per cent and temperature of 25±1ºC. Leaves were observed for the development of initial symptoms at regular intervals after inoculation. The incubation period (p) taken for the appearance of the disease was recorded as explained below:

$$p = t_s - t_i$$

Where,

p	=	incubation period in hours
t_i	=	time of inoculation of leaves
t_s	=	time of appearance of disease symptoms

3.3 MORPHOLOGICAL CHARACTERS OF THE PATHOGEN

The morphological characters of the test fungus were studied on the pathogen culture grown on PDA medium. Seven-day old pathogen culture was used for studying morphological characters. Observations on colony color, hyphal color and septation diameter, conidial shape, size (length and breadth) and the number of transverse and longitudinal septa in conidia were recorded using Olympus Microscope and Promagnus Software.

3.4 DISEASE MANAGEMENT

3.4.1 *In vitro* evaluation

A) *In vitro* evaluation of botanicals against *Alternaria brassicicola* causing leaf spots in cauliflower

About 2 months old freshly harvested 200 g leaves each of Neem (*Azadirachta indica*), Tulsi (*Ocimum sanctum*), Aloe (*Aloe vera*), Karvaya (*Roylea elegans*), Lemon grass (*Cymbopogon citratus*), Pudina (*Mentha piperita*), Banna (*Vitex nigundo*), Bougainvillea (*Bougainvillea glabra*) and Darek (*Melia azedarach*) (Table 1) were taken for evaluation of botanicals, against the test pathogen. Specified quantity (200g) of leaves/seeds of each plant were taken and then washed thoroughly with fresh water and finally rinsed with sterilized distilled water. Samples belonging to each plant were grinded in mixer and blender by adding small quantity of sterilized distilled water so that fine paste is made. After grinding, a total volume of 200 ml distilled water was added and homogenized in orbital shaker at the rate of 2000 rpm for half an hour to get 50 per cent extract of each plant. The plant material was then filtered through a double-layered muslin cloth in 500ml conical flask and plugged in with non-absorbent cotton. Sterilization of the filtered extract of different plants was done in autoclave at 5 psi pressure for 30 minutes for 3 consecutive days and then the extracts were kept in refrigerator for further use.

Evaluation of botanical extracts was done by poisoned food technique (Flack, 1907). Botanical extracts were evaluated at 10 per cent concentration by incorporating 20.0 ml of sterilized extract of botanicals (50%) in 80 ml of sterilized PDA medium in separate conical flasks. The poisoned medium was poured in the sterilized Petri plates under aseptic conditions. The Petri plates were inoculated aseptically after solidification of the medium. For this, 5mm diameter mycelial disc of the pathogen was cut aseptically with cork borer from 10 days old pure culture of *Alternaria brassicicola* and put in the centre of the Petri plate containing the PDA medium poisoned with the botanical extracts. Separate control plates of PDA medium were also inoculated without poisoning with the botanicals to evaluate the relative *in vitro* efficacy of botanicals. Four replications of each treatment were kept and the Petri plates were incubated at 25±1ºC temperature in BOD incubator till the control plates were fully covered with the fungal mycelium. Inoculated plates were observed daily and the colony diameter of the test pathogen was recorded till the control plates were full with mycelium of the test pathogen. The per cent mycelial growth inhibition was calculated according to the formula given by (Vincent, 1947).

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

I = Percent mycelial growth inhibition

C = Linear growth in control (mm)

T = Linear growth in treatment (mm)

Table 1. Details of the plants used for making botanicals formulations:

S. No.	Common name	Botanical name	Plant part used
1.	Neem	*Azadirachta indica* L.	Leaves
2.	Tulsi	*Ocimum sanctum* L.	Leaves
3.	Aloe	*Aloe vera* L.	Leaves
4.	Karvaya	*Roylea elegans*	Leaves
5.	Lemon grass	*Cymbopogon citratus*	Leaves
6.	Mint	*Mentha piperita* L.	Leaves
7.	Banna	*Vitex nigundo*	Leaves
8.	Bougainvillea	*Bougainvillea glabra*	Leaves
9.	Darek	*Melia azedarach*	Seeds/Leaves

B) *In vitro* evaluation of different isolates of yeast against *Alternaria brassicicola*

Dual culture method

Bio-control activity of yeasts isolates from different sources was evaluated *in vitro* for their antagonism against *Alternaria brassicicola* by dual culture technique (Huang and Hoes, 1976). In this experiment, six isolates of yeasts and the test pathogen- *Alternaria brassicicola* from cauliflower were separately grown on PDA medium. First, sterilized PDA medium (20ml) was poured aseptically into sterilized Petri plates. Petri plates were then inoculated by placing mycelial disc of 5 mm diameter, which were cut aseptically with the help of cork borer from ten days old actively growing culture, of *Alternaria brassicicola* to one side of the Petri plate. In the same way the yeast isolates were inoculated by streaking exactly on the other side of the same Petri plate by leaving 3-4 cm gap between the inoculations of the antagonists and the pathogen (Afsah-Hejri, 2013). For comparison, the test pathogen-*Alternaria brassicicola* was grown alone in Petri plates. These plates were incubated in BOD incubator at 25$\pm$2°C. Each treatment was replicated four times and inhibition zone was recorded till the test pathogen completely covered the control plates. The per cent mycelial growth inhibition was calculated by the formula (Vincent 1947) given as below:

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

I	=	Percent mycelial growth inhibition
C	=	Linear growth in control (mm)
T	=	Linear growth in treatment (mm)

C) *In vitro* evaluation of botanicals and yeasts based bio-formulations against *Alternaria brassicicola*

Two Field Formulations were made by selecting 5 effective anti-fungal plants. To make these formulations, equal quantity of leaves or seeds of each plant species were taken and then the equal quantity of water (Field Formulations 1) or cow urine (Field Formulations 2) on weight: volume basis was added to make these two different formulations. Other bio-formulations were made by combining Field Formulations (1 and 2) with the two effective anti-fungal yeasts and the natural sources of yeasts-butter milk. These bio-formulations were compared with the Field Formulations 1 and 2, effective yeasts and the recommended fungicides mancozeb and copper oxychloride as per the detail of the treatments in Table 2. These were evaluated *in vitro* for their efficacy on mycelial growth of *Alternaria brassicae/ A.brassicicola* by Poison Food Technique (Flack, 1907; Nene and Thapliyal, 1979).

To make the stocks of different treatments for the food poisoning technique, double strength PDA was made and in each treatment 50ml double strength PDA medium was taken in a conical flask (150ml) which were sterilized at 15 psi (1.05 kg/cm^2) pressure at 121.6ºC for 20 minutes. Simultaneously, concentration of different treatments were prepared in 50ml i.e. the equal amount of sterilized distilled water was mixed in order to get the desired concentration after mixing the solution in the double strength PDA media. For the preparation of 10% concentration of Treatment 1 and 2, 10 ml of Yeast 1 and Yeast 2 broth was mixed in

40ml of sterilized distilled water. The medium was poured in sterilized Petri plates under aseptic conditions. The Petri plates were inoculated in the centre with a culture disc of 5mm diameter of a fresh culture of the test pathogen. A control was also maintained in which only plain sterilized water was added to the double strength PDA media.

Each treatment was replicated three times and the inoculated plates were incubated at 25 ± 1ºC in BOD incubator. Inoculated plates were observed daily and the colony diameter of the test pathogen was recorded till the control plates were full with mycelium of the test pathogen. The per cent mycelial growth inhibition was calculated by the formula given by (Vincent, 1947).

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

I	=	Percent mycelial growth inhibition
C	=	Linear growth in control (mm)
T	=	Linear growth in treatment (mm)

Table 2. Detail of the treatments of Yeasts, Field Formulations and Bio-formulations evaluated for their anti-fungal activities

Treatment No.	Treatment details
T1	Yeast BK 5
T2	Yeast BK 7
T3	Field formulation 1 (Water)
T4	Field formulation 2 (Cow urine)
T5	Butter milk
T6	Field Formulation 1 + Yeast BK 5
T7	Field Formulation 1 + Yeast BK 7
T8	Field Formulation 1 + Butter milk
T9	Field Formulation 2 + Yeast BK 5
T10	Field formulation 2 + Yeast BK 7
T11	Field formulation 2 + Butter milk
T12	Mancozeb
T13	Copper oxychloride

D) Evaluation of botanicals and yeast-based formulations against Alternaria leaf spots of cauliflower under field conditions

Different botanicals, yeast-based formulations and fungicides were evaluated against Alternaria leaf spot of cauliflower under field conditions in Randomized Block Design (RBD) at experimental farm of Department of Plant Pathology, UHF, Nauni where Alternaria leaf spot disease had been appearing in the previous cropping seasons.

Healthy seedlings of cauliflower cultivar "Pusa Snowball K1" were transplanted in different plots of size 2 m × 2 m at a spacing of 60 cm × 45 cm during the last week of October 2020. Eight treatments including control were replicated five times and recommended intercultural operations were followed to raise a healthy crop. Five periodic sprays of different botanicals, yeast-based formulations and fungicides were given at 10 days interval starting with the initiation of the disease.

Table 3. Detail of the treatments of Yeasts, Field Formulations and Bio-formulations evaluated for their anti-fungal activities in the field:

Treatment No.	Detail of the treatment
T1	Field formulation 2
T2	Field Formulation 2 + Yeast BK 5
T3	Field Formulation 2 + Yeast BK 7
T4	Field Formulation 2 + Butter milk
T5	Butter milk
T6	Mancozeb
T7	Copper oxychloride

a) Recording of disease severity:

The disease severity was recorded on ten leaves of randomly selected cauliflower plants per replication at 60 DAT and the severity was scored using 0-4 point scale suggested by (Mayee and Datar, 1986).

Rating value	Nature of infection
0	No infection (healthy)
1	1-25% leaf area infected
2	25.1-50% leaf area infected
3	50.1-75% leaf area infected
4	>75% leaf area infected

The per cent disease severity was worked out according to McKinney (1923) as given below:

$$(\%)\text{ Disease severity} = \frac{\text{Sum of all disease ratings}}{\text{Total number of ratings} \times \text{Maximum disease grade}} \text{ X } 100$$

b) Recording of disease incidence :

The disease incidence was recorded at 60 DAT on the basis of characteristic symptoms of the disease by using the following formula:

$$(\%)\ \text{Disease incidence} = \frac{\text{Number of infected plants}}{\text{Total number of plants assesed}} \times 100$$

c) Crop yield:

Crop yield was recorded in each of the treatment plots including that of control treatment to work out the treatment response in terms of crop yield.

3.5 MICROBIAL COUNT

As the different treatments were combinations of constituents from the plants and yeasts, experiment was conducted to know the microbial count of the different treatment combinations. Serial Dilution Technique was followed for evaluation of microbial count from liquid samples which were used as treatments. Three different media viz. Potato Dextrose Agar Medium (PDA), Yeast Peptone Dextrose Agar Medium (YPDA) and Nutrient Agar Medium (NA) were used for isolation of fungal, actinomycetal and bacterial bioagents, respectively. Streptocycline was added in PDA and YPDA to check the growth of bacterial contaminants. Dilution blanks were prepared and labelled as 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, $10^{-7}$and 10^{-8}. Stock solution of different samples were prepared by adding 1ml liquid sample into dilution blank containing 9 ml of sterilized distilled water. Mixture is thoroughly homogenised by rolling the tube back and forth between hands for uniform distribution of organisms. From the stock suspension, 1 ml suspension was transferred to a known volume of dilution blank 10^{-1} by using 5 ml sterilized pipette and homogenised. From the suspension, 10^{-1}, 1 ml was transferred to a known volume of distilled water in dilution blank 10^{-2}. This procedure was repeated till the original sample has been diluted 100,000,000 (10^{-8}) times. From the stock solutions with dilutions of 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 100 micro litre of suspension were taken and poured on the Petri plates containing PDA and YPDA media for getting the count of actinomycetes and fungi. Whereas from the stock solutions of different treatments with dilutions of 10^{-6}, 10^{-7}, 10^{-8}, 100 micro litre of suspension from each were poured to find the count of different bacteria in the treatments. The plates were incubated at 25±1°C for 3 to 5 days and were observed for appearance of colonies from 3rd to 5th day.

3.5.1 Enumeration of microflora count of the treatments

Microbial count in the different treatments was calculated by counting the number of viable colonies and is represented by colony forming units (cfu).

$$\text{Cfu/ml} = \frac{\text{Number of colonies obtained} \times \text{Reciprocal of dilution used}}{\text{Volume of sample used}}$$

Individual colonies of fungi, bacteria and actinomycetes were grouped and selected based on morphological / cultural characteristics (Seeley and VanDemark, 1975).

3.6 STATISTICAL ANALYSIS:

The data to be recorded will be analysed using MS-excel and OPSTAT as per the design of experiment for working out the following values:

Analysis of variance

Analysis of variance shall be done as suggested by Gomez and Gomez (1984):

Source of variation	**Degree of freedom**	**Sum of square**	**Mean sum of square**	**Fcal**
Replication	r-1	Sr	$\frac{\text{Sr}}{(\text{r}-1)} = \text{Mr}$	$\frac{Mr}{Me}$
Treatment	t-1	St	$\frac{\text{St}}{(\text{t}-1)} = \text{Mt}$	$\frac{Mt}{Me}$
Error	(r-1)(t-1)	Se	$\frac{Se}{(r-1)(t-1)} = Me$	
Total	(rt-1)	S_T		

where,

R	=	Number of replications
t	=	Number of treatments
Sr	=	Sum of square due to replications
St	=	Sum of square due to treatments
Se	=	Sum of square due to error
S_T	=	Total sum of squares
Mr	=	Mean sum of square due to replications
Mt	=	Mean sum of square due to treatments
Me	=	Mean sum of square due to error

The replication and treatment mean sum of square will be tested against mean sum of square due to error by 'F-test' for (r-1), (r-1) (t-1) and (t-1), (r-1) (t-1) degree of freedom for RBD at 0.05 per cent level of significance.

The calculated F-values will be compared with tabulated F-value. When F-test will be found significant, critical difference will be calculated to find out the superiority of one treatment over the others.

The standard error and critical differences will be calculated as follows:

The replication and treatment mean sum of square shall be tested against error mean squares by 'F' test at (t-1), (t) (r-1) degree of freedom for CRD at 0.05 per cent level of significance.

The calculated F-values will be compared with tabulated F-value. When F-test will be found significant, critical difference will be calculated to find out the superiority of one treatment over the others.

The standard error and critical differences will be calculated as follows:

SE (m) ±	=	$\sqrt{\mathrm{Me/r}}$
SE (d) ±	=	$\sqrt{\mathrm{2Me/r}}$
$CD_{0.05}$	=	SE (d) x t(0.05) (t) (r-1) df
SE (m) ±	=	Standard error of mean
SE (d) ±	=	Standard error of differences
$CD_{0.05}$	=	Critical difference at 5% level of significance

Chapter-4

RESULTS AND DISCUSSION

The results of the present investigations have been described under the following headings:

4.1 Isolation and Identification

 4.1.1 Isolation and Identification of the pathogen

 4.1.2 Isolation and Identification of yeasts

4.2 Pathogenicity studies of the pathogen(s)

4.3 Morphological characters

4.4 Disease management

4.5 Microbial count of bio-formulations

4.1 ISOLATION AND IDENTIFICATION

4.1.1 Isolation and Identification of the Pathogen

Isolation

The isolation of the test fungus associated with Alternaria leaf spots of cauliflower was made from the samples of infected leaves collected from the experimental farm, Department of Plant Pathology, Nauni, Solan. Cauliflower leaves showing characteristic symptoms were brought to laboratory and the associated pathogen was isolated by placing surface-sterilized bits of infected leaves on Potato Dextrose Agar medium (PDA) plates. Fungus associated with the infected leaves protruded out of the infected bits in case of most of the bits cultured on Petri plates and which appeared as dull white mycelial growth. The fungus grew profusely on PDA medium with white mycelium at 25±1 °C temperature. The isolated fungus was purified by hyphal tip method. The purity of isolated fungus was regularly maintained by sub-culturing and the culture was preserved at 4±1ºC in the refrigerator.

Potato Dextrose Agar media (PDA) was found to be most suitable culture media for the growth of the pathogen which was later identified as *Alternaria brassicicola*. Ansari *et al.* (1989) and Kumar and Singh (2003) also reported PDA medium was best suited for the growth of *Alternaria brassicae* in comparison to Czpex Dox Agar and Corn Meal Agar media. Charith *et al.* (2020) also reported the suitability of PDA for higher growth of this pathogen.

Identification

The pure culture of the pathogen obtained from the diseased leaves exhibiting characteristic symptoms of Alternaria leaf spots was examined visually followed by microscopic examination. On the basis of cultural and morphological (hyphal and conidial) characters, the test fungus was identified as *Alternaria brassicicola* (Schwein.) Wiltshire.

In the initial stages of growth, the culture of test fungus was olive green in colour with smooth margins and distinct zonations, which later turned olivaceous-brown to greenish-black in colour with light brown to white periphery. The microscopic studies indicated that the mycelium was septate and profusely branched; hyaline initially which then turned to brown or olivaceous brown. The conidiophores were olivaceous, septate and irregularly branched with a size of 35-45 × 5-8 µm. The conidia were dark, cylindrical to oblong in shape and are muriform with a size of 44-55 × 11-16 µm. A varying number of longitudinal and transverse septa were observed in different conidia.

On the basis of the morphological characters and their analogy with that given by Wiltshire (1947), it was identified as *Alternaria brassicicola* (Schwein.) Wiltshire. The characteristics of the *Alternaria* species described by other researchers in the literature (Ellis, 1971; Simmons, 2007; Meena *et al.,* 2010). The fungus is also reported to cause leaf spot disease on the same crop in association with another fungus *Alternaria brassicae* (Singh, 1987).

Ellis (1971) while conducting morphological studies on *Alternaria brassicicola* observed the colonies of the pathogen as amphigenous, effused, dark olivaceous brown to dark blackish brown and velvety. Mycelium was reported to be immersed, branched with septate hyphae; hyaline at first, later turning to brown or olivaceous brown, inter and intra-cellular, smooth, 1.5-7.5 µm in thickness. Humpherson-Jones and Phelps (1989) reported temperature range of 18-24°C optimum for sporulation of *Alternaria brassicae* and 20-30°C for *Alternaria brassicicola.* In *Alternaria,* morphological characteristics of conidia and conidiophores and sometimes host plant association, provide the major taxonomic criteria for delimitation of fungal species (David, 1991).

The identification of the test fungus was further reconfirmed as *Alternaria brassicicola* (Schwein.) Wiltshire with the assistance of Mycologist, Dr. PN Chowdhry, National Centre of Fungal Taxonomy, New Delhi with ID No. 10014.21 dated 07/03/2021.

4.1.2 Isolation and Identification of Yeasts

Isolation

A total of six isolates were isolated from different sources like fruits, raisins, etc. Out of these six, five yeast isolates i.e. BK1, BK2, BK4, BK5, BK6 were procured from Dr. Sanjeev Kumar Verma, VCSG Uttarakhand University of Horticulture and Forestry, Pauri, Uttarakhand and BK 7 was isolated in the Department of Plant Pathology, Nauni, Solan. All the yeast isolates grew profusely on YPDA medium with white or creamy growth at 25±1 °C temperature. The purity of isolated yeasts was regularly maintained by sub-culturing and the cultures were preserved at 4±1ºC in the refrigerator.

Identification

Pure cultures of different isolates of yeasts used in present study were examined visually followed by microscopic examination. On the basis of cultural and morphological characters, all the yeasts were identified as *Saccharomyces cerevisiae* Meyen ex E.C. Hansen. The cultural study indicated that the yeasts produced white; creamy pigmentation, oblong/ eclipse colony with cell length of 2-8 µm and cell breadth of 2-5 µm and attained a full growth of 90 mm on Yeast Peptone Dextrose Agar medium at 25±1 ^{0}C in 3-4 days. Similar morphological and cultural characters were described by Hartwell, 1974; Himankshi, 2011; Reis *et al.,* 2014. The identification of the yeasts were further reconfirmed as *Saccharomyces cerevisiae* Meyen ex E.C. Hansen with the help of Dr. PN Chowdhry at National Centre of Fungal Taxonomy, New Delhi with ID No. 10008.21, 10009.21, 10010.21, 10011.21, 10012.21 and 10013.21 dated 07/03/2021.

4.2 PATHOGENICITY STUDIES OF THE PATHOGEN(S)

4.2.1 Incubation period

The observations on incubation period (h) and symptom development following inoculation of potted plants and detached leaf were recorded and presented in Table 1. Pathogenicity was also proved through detached leaf technique as well. Observations on the incubation period and symptom development were recorded in each case and have been presented in Table 1.

In the detached leaves technique, the initial symptoms of the disease appeared as small yellow fleck with light brown centre after 62.1 hours of inoculation. While in the other method, the initial symptoms on the leaves of the potted plant appeared after 120.3 hours of inoculation as small, isolated scattered pale yellow to brown spot with a yellow halo. The fungus was consistently re-isolated from lesions in the inoculated plants and identified as *Alternaria brassicicola* thereby proving the pathogenicity of the pathogen according to Koch's postulates.

In other such studies, the pathogenicity of different isolates of *A. brassicicola* and *A. brassicae* on Brassica hosts had been proved by many workers (Conn and Tiwari, 1989; Sharma *et al.,* 2002; Iacomi-Vasilescu *et al.,* 2003; Doullah *et al.,* 2006; Deep and Sharma, 2012 and Siciliano *et al.,* 2017). Doullah *et al.* (2006) tested the pathogenicity of *Alternaria brassicicola* on *Brassica rapa* through three different inoculation methods. They discovered that the detached leaf inoculation method was the most efficient one, with symptoms appearing within 24 hours of inoculation of the fourth true leaves. The findings of the present pathogenicity studies conducted on detached leaves are also in accordance with the findings of Deep and Sharma (2012). While proving the pathogenicity of *Alternaria brassiciola* and *Alternaria brassicae* on cauliflower, injury was made on the surface with a fine needle by pinprick method and symptoms were observed on leaves third day after inoculation in the inoculated leaves. Siciliano *et al.* (2017) proved the pathogenicity of *Alternaria* spp. causing leaf spot on cabbage and cauliflower plants by raising them in pots for two months. The plants were inoculated with conidial suspension at $1\text{-}5\times10^5$ conidia per ml using atomizer.

Firstly, *Alternaria* symptoms were observed on old leaves. Kumar (2017) also reported that early blight symptoms can be observed starting from three days after inoculation under controlled conditions.

4.2.2 Symptom Development

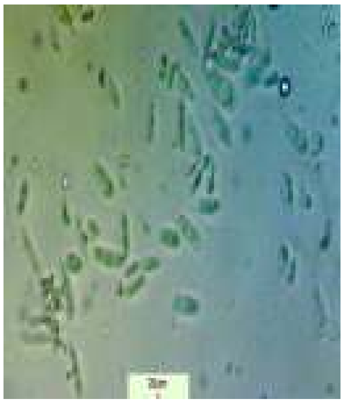
a) On potted plants

Initial symptoms observed on the leaves of potted plants were small, isolated scattered pale yellow to brown spots with a yellow halo that grew in size and became black necrotic circular to irregular in form. Blighting of leaves occurs as spots coalesced.

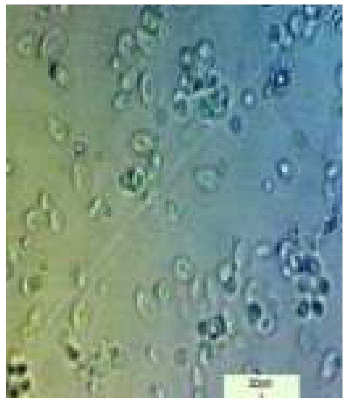
S.cerevisiae (BK1- chickpea)

S.cerevisiae (BK2-apple pomace)

S.cerevisiae (BK4- chickpea)



BK 1

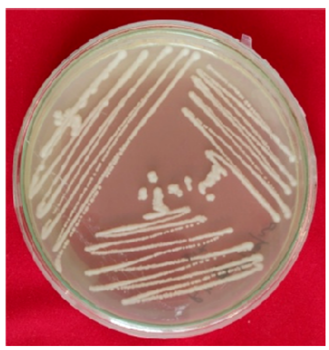


BK 2

BK 4

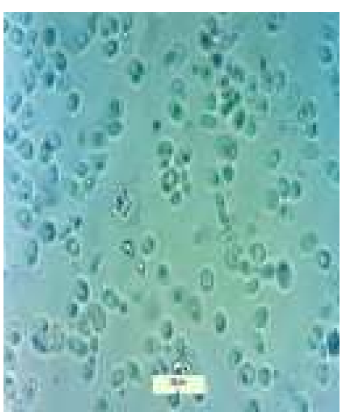
Microphotographs of spores

S.cerevisiae (BK5- Grape)



S.cerevisiae (BK6- Grape)

S.cerevisiae (BK7- Raisin)



BK 5

BK 6

BK 7

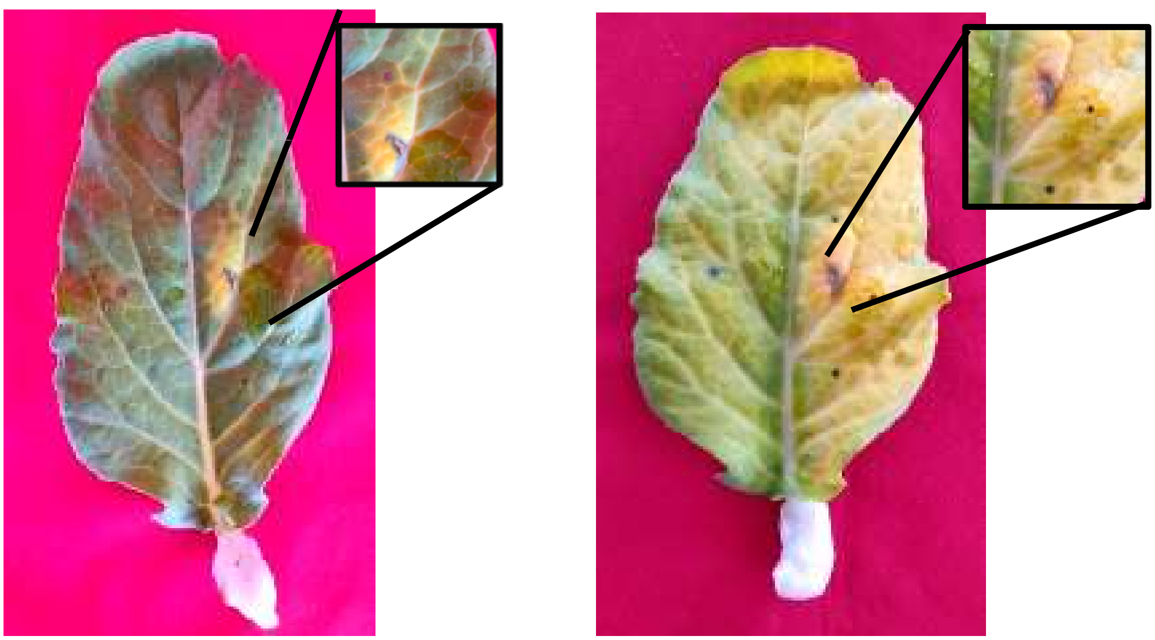
Microphotographs of spores

Plate 1. Different isolates of yeast-*Saccharomyces cerevisiae*

Inoculated plants **Control** **Control** **Inoculated plants**

Initial symptoms **Symptoms at later stages**

A. Pathogenicity experiment in pots



Initial stages **Later stages**

B. Pathogenicity experiment with detached leaves

Plate 2. Proving pathogenicity of Alternaria leaf spot (*Alternaria brassicicola)*

Table 1. Pathogenicity of *Alternaria brassicicola* on cauliflower plant and plant parts

PATHOGENICITY TEST			
Plant/plant parts			**Incubation period (h)**
Potted plants			120.30 hours
Detached leaves			62.10 hours
Symptom Development	**On potted Plants**		The initial symptoms on leaves appeared as small, isolated scattered pale yellow to brown spot with a yellow halo which further increase in size and become black necrotic circular to irregular in shape. Blighting of leaves occur with the coalescing of spots.
	On detached leaves	**Initial stages**	Appeared as small yellow fleck with light brown centre.
		Later stages	Yellow fleck turned into brown necrotic irregular to circular spots surrounded by a yellow halo.
		Advanced Stages	The necrotic spots enlarged in size covering the whole leaf lamina giving blighted appearance accomplished by sporulation.

b) Detached leaves

The initial symptoms recorded on leaves appeared as small yellow fleck with light brown centre which further turned into yellow to brown dark spots with a yellow halo which further increased in size and became black necrotic circular to irregular in form. Blighting of leaves occurred at the later stages with the coalescing of spots. In advanced stages, the necrotic spots enlarged in size covering the whole leaf lamina giving them blighted appearance and the blighted portion had abundance of sporulation.

4.3 MORPHOLOGICAL CHARACTERS

The morphological characters of the test pathogen (*Alternaria brassicicola*) were studied and the description has been given in Table 2. Identification of the pure culture of isolated pathogen was carried out on the basis of morphological characteristics such as color and growth of mycelial colony, septation and diameter of hyphae, shape and size of conidiophores and conidia.

Mycelial colonies of isolated fungus were found to be brown to olivaceous brown in color with fluffy or cottony dense aerial growth. The cultural study indicated that the pathogen produced olivaceous brown to greenish black in colour, with light brown to white periphery, cottony-fluffy growth and the mycelium attained a full growth of 90 mm on PDA medium at 25±1^{0}C in 9 days.

Mycelium

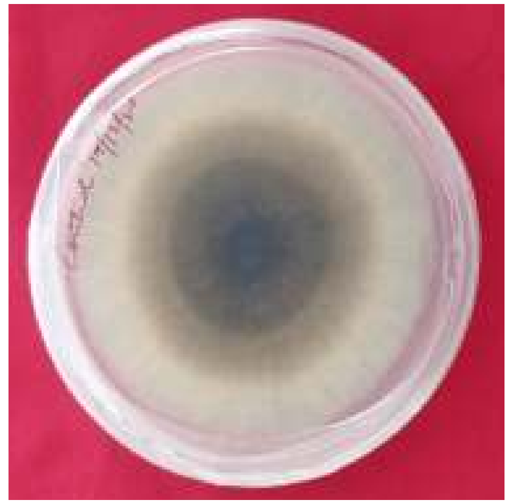
In the initial stages of growth, the colony colour was dull white in appearance which later turned to olivaceous brown to greenish black in colour. Initially, the mycelium strands were hyaline in colour which later turned to brown or olivaceous brown when grew older. The hyphae was septate and profusely branched with a diameter ranging from 2.5-6.02 µm with an average diameter of 4.26 µm.

Conidia

Microscopic studies (400X magnification) indicated that the conidiophores were olivaceous, septate and branched (Table 2). Conidia of *Alternaria brassicicola* were dark, cylindrical to oblong in shape and muriform with no beak formation. Size of conidia ranged from 44-55 × 11-16 µm with average size of 49.5 × 13.5 µm and were having 5-8 transverse and 0-5 longitudinal septa, respectively. On the basis of above mentioned morphological characteristics, the isolated pathogen was identified as *Alternaria brassicicola* (Schwein.) Wiltshire, as the similar morphological characters have been documented in "Illustrated Genera of Imperfect Fungi" (Barnett, 1955) and described in "Monograph of the Fungus Genus *Alternaria*".

In present investigation, the mycelium of the pathogen was olive grey to greyish black and septate. The detailed morphological characters of this pathogen recorded are represented in Table 2. Kolte (1985) in his studies on this fungus (*A. brassicicola*) reported that mycelium is septate, olive grey to greyish black in colour; the conidiophores are olivaceous, septate, branched, measuring 35-45 µm in length and 5-8 µm in width and the conidia are dark, cylindrical to oblong, muriform without beak measuring 44-55 µm in length and 11-16 µm in width with 5-8 transverse and 0-4 longitudinal septa. He further elaborated that the fungus grows faster in media with high sporulation and appears as well developed black sooty colony with distinct zonations. Kumar *et al.* (2014) also reported similar morphological characteristics of *Alternaria brassicicola*.

Dorsal side



Ventral side

A. Pure culture of *Alternaria brassicicola* on PDA

Mycelial mat of *A. brassicicola*

Hyphae of *A. brassicicola*

Conidia of *A. brassicicola*

Septa formation in *A. brassicicola*

B. Microphotographs of *Alternaria brassicicola*

Plate 3. Morphological characters of *Alternaria brassicicola*

Table 2. Morphological characters of *Alternaria brassicicola* causing leaf spot of cauliflower

<table>
<tr><th colspan="2">Morphological character(s)</th><th>Description</th></tr>
<tr><td rowspan="2">Mycelium Colour</td><td>Young (3 days)</td><td>Dull white with smooth growth</td></tr>
<tr><td>Old (9 days)</td><td>Olivaceous brown to greenish black in colour with light brown to white periphery</td></tr>
<tr><td colspan="2">Hyphae / Mycelium</td><td>Septate, brown or olivaceous brown, profusely branched</td></tr>
<tr><td colspan="2">Conidiophore</td><td>Olivaceous, septate, branched, measuring 35-45µm in length and 5-8 µm in width</td></tr>
<tr><td colspan="2">Conidial shape</td><td>Dark, cylindrical to oblong in shape and are muriform, brownish in colour.</td></tr>
<tr><td rowspan="2">Conidia size</td><td>Length of conidia</td><td>44-55 µm</td></tr>
<tr><td>Width of conidia</td><td>11-16 µm</td></tr>
<tr><td rowspan="2">Conidial septation</td><td>Number of transverse septa</td><td>5-8</td></tr>
<tr><td>Number of longitudinal septa</td><td>0-5</td></tr>
</table>

Same morphological characters like colony colour, growth habit, conidia shape, size and septation were also described by Bedi and Singh, 1972, Grogan *et al.,* 1975 and Balasubramanian, 1979.

4.4 DISEASE MANAGEMENT:

In this study, efforts have been made to develop alternative disease management strategy based on antagonistic yeasts and botanicals after evaluating them under both *in vitro* and (*in vivo*) field conditions.

4.4.1 *In vitro* evaluation

A) *In vitro* efficacy of aqueous extract of different botanicals against mycelial growth of *Alternaria brassicicola*

Aqueous extract of leaves from nine different plants namely; Neem (*Azadirachta indica*), Tulsi (*Ocimum sanctum*), Aloe (*Aloe vera*), Karvaya (*Roylea elegans*), Lemon grass (*Cymbopogon citratus*), Pudina (*Mentha piperita*), Banna (*Vitex nigundo*), Bougainvillea (*Bougainvillea glabra*) and Darek (*Melia azedarach*) were evaluated at 10% concentration under *in vitro* condition against the test pathogen (*Alternaria brassicicola*) for their inhibitory effect on mycelial growth by poisoned food technique (Flack, 1907) and results on per cent mycelial inhibition are presented in Table 3.

All the botanicals significantly reduced the mycelial growth of the pathogen in comparison to control. Among these nine plants, karvaya leaves extract proved highly effective followed by neem in reducing the mycelial inhibition i.e. 52.64 and 51.11 per cent, respectively in comparison to control but both were found significantly at par. Lemon grass extract was found least effective with mycelia inhibition of 36.39 per cent. These results are in agreement with the findings of Sharma *et al.* (2007) who reported that neem leaf extract resulted in 43.3 and 26.7 per cent inhibition of the mycelia growth of *Alternaria solani* at 0.1 and 0.01 concentration, respectively. Sasode *et al.* (2012) reported the effectiveness of Neem, Eucalyptus, Datura, Lantana, Tulsi and Pudina at 10 per cent concentration against *Alternaria brassicae* and *Alternaria brassicicola* infecting mustard under *in vitro* conditions. Among five plant extracts, *Azadirachta indica* has been reported significantly superior followed by *Allium sativum*, *Parthenium hysterophorus* and *Datura stramonium* (Raza *et al.,* 2015).

Table 3. *In vitro* efficacy of different botanicals at 10 per cent concentration against mycelial inhibition of *Alternaria brassicicola* causing leaf spot of cauliflower

Extract of the Botanical(s)	Per cent mycelial inhibition
Neem (*Azadirachta indica* L.)	51.11 (45.62)*
Tulsi (*Ocimum sanctum* L.)	45.97 (42.67)
Aloe *(Aloe vera* L.)	46.67 (43.07)
Karvaya (*Roylea elegans*)	52.64 (46.49)
Lemon grass *(Cymbopogon citratus*)	36.39 (37.08)
Mint *(Mentha piperita* L.)	40.28 (39.38)
Banna (*Vitex nigundo*)	36.95 (37.42)
Bougainvillea (*B. glabra*)	40.28 (39.38)
Darek (*Melia azedarach*)	40.97 (39.78)
Control	0.00 (0.00)
$CD_{0.05}$	1.35
SE(m)	0.46
SE(d)	0.66

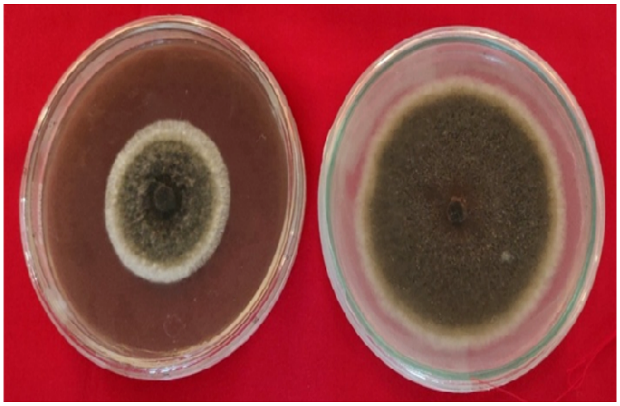
*Figures in parenthesis are arc sign transformed values

Neem Tulsi Aloe vera

Karvaya Lemon grass Mint

Darek Bougainvillea Banna

Plate 4. Aqueous extracts of plants evaluated



Karvaya

Neem

Aloe vera

Tulsi

Darek

Plate 5A. *In vitro* mycelial inhibition of *Alternaria brassicicola* with aqueous extracts of different botanicals

Bougainvillea

Mint

Lemon grass

Banna

Plate 5B. ***In vitro*** **mycelial inhibition of** ***Alternaria brassicicola*** **with aqueous extracts of different botanicals**

Overall results demonstrated that all the tested concentrations of *Azadirachta indica* were found significantly effective for controlling early blight of tomato. Gupta *et al.* (2019) also found that the neem plant extract was found highly effective against pathogen at 15 and 25 per cent with mycelial inhibition of 65.55 and 68.88 per cent, respectively. Chamoli *et al.* (2020) reported maximum mycelium growth inhibition of *Alternaria brassicicola* in neem leaf extract followed by garlic, tulsi and lemon grass.

Plants produce metabolites which have antifungal activities. The effectiveness of leaf extract of different botanicals in the present study can be corroborated due to presence of one or the other acid or organic molecules. Qurecetin and β-sitosterol, polyphenolic flavonoids, produced in neem were known to have antibacterial and antifungal properties against many pathogenic fungi (Alzohairy, 2016).

B) *In vitro* evaluation of different isolates of yeast against mycelial growth of *Alternaria brassicicola*

Six yeast isolates were evaluated for their inhibitory effect on mycelial growth of *Alternaria brassicicola* by dual culture technique. The results on per cent mycelial inhibition are presented in Table 4.

Table 4. *In vitro* efficacy of different isolates of yeasts against mycelial growth of *Alternaria brassicicola*

Yeast Isolate	Per cent mycelial growth inhibition
BK 1	43.61 (41.31)*
BK 2	53.61 (47.05)
BK 4	55 (47.85)
BK 5	59.72 (50.59)
BK 6	53.05 (46.73)
BK 7	55.56 (48.17)
Control	0.00 (0.00)
$CD_{0.05}$	1.68
SE(m)	0.57
SE(d)	0.80

*Figures in parenthesis are arc sign transformed values

All the yeast isolates significantly reduced the mycelial growth of the pathogen in comparison to control. Yeast isolate BK 5 was found most effective significantly superior to other treatment with 59.72 per cent mycelial inhibition of the pathogen followed by BK 7 which reduced the mycelia growth of the pathogen by 55.56 per cent. While minimum mycelial inhibition of 43.61 per cent was recorded in isolate BK 1, efficacy of other isolates (BK 2, BK 4 and BK 6) was in between. Sharma *et al.* (2009) reported the antagonistic properties of yeasts (*Candida oleophila*, *Candida sake*, *Metschnikowia fructicola*, *Aureobasidium pullulans*, *Saccharomyces cerevisiae*, and *Cryptococcus*) against *Alternaria* and other fungal pathogens like *Botrytis, Penicillium, Rhizopus, Colletotrichum, Monilinia, Aspergillus* under *in vitro* conditions. Hu *et al.* (2015) reported significant inhibition of *Penicillium expansum* causing blue mold of pear with the application of yeasts and the mechanism of action being mycoparasitism. Prendes *et al.* (2018) were the first to report the action of yeast against the *Alternaria alternata* pathogen on wine grape berries. Anuj (2019) reported efficacy of three yeast isolates against *Alternaria* out of 23 evaluated and highest inhibition of 38.7 per cent was found in the isolate from green grape fruits.

Other researchers have also reported the efficacy of yeasts against different plant pathogenic fungi (Li *et al.*, 2016; Ferraz *et al.,* 2019; Cordero-Buesco *et al.,* 2017; Nally *et al.*, 2018).

C) *In vitro* evaluation of field formulations of effective botanicals and their combinations with effective yeasts against mycelial growth of *Alternaria brassicicola*

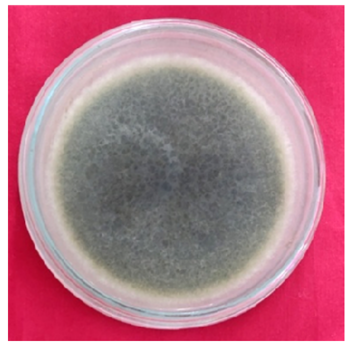
Perusal of data (Table 5) indicated that among different treatments, individual treatment with yeast- BK5 and mancozeb were found most effective and statistically at par with 94.07 per cent average inhibition in mycelial growth of the pathogen followed by butter milk with 92.96 per cent average inhibition in mycelial growth of the pathogen in comparison to control. Minimum per cent growth inhibition of 47.78 per cent was reported in Field formulation 1. The interesting part of the study is that making of bio-formulations by combining other treatments with yeast- BK5 and butter milk did not enhance the efficacy of the combinations (Table 5). Instead, the efficacy of such treatments from T6 to T11 was reduced which indicate that the active ingredients in Field Formulations 1 and 2 may have exerted inhibitory effect on the active ingredients of yeasts present in the Treatments T1 and T5.

Isolates of *Candida* sp. have been reported to be effective against several fungal pathogens (Guinebretiere *et al.,* 2000; Zahavi *et al.,* 2000; Bleve *et al.,* 2006).


BK 5


BK 7


BK 4


Comparative growth of the pathogen in control


BK 2


BK 6


BK 1

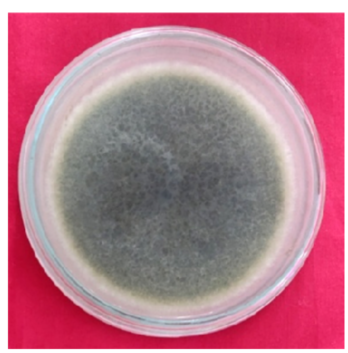

Comparative growth of the pathogen in control

Plate 6. *In vitro* efficacy of different isolates of yeasts in causing mycelial inhibition of *Alternaria brassicicola*

In earlier studies, cow urine and butter milk when used alone reduced the mycelial growth of *Rhizoctonia bataticola* as butter milk contains *Lactobacillus, Enterococcus, Lactococcus, Bacillus* and *Saccharomyces* species. Sundaraj *et al.* (1998) reported that butter milk at 500 and 1000 ppm completely inhibited the mycelial growth of *Rhizoctonia bataticola* and *Rhizoctonia solani* on PDA amended media. Similar findings of efficacy of butter milk was reported against *Macrophomina phaseolina* in which size and number of sclerotia was reduced in cow urine and butter milk amended media (Raja and Kurucheve, 1997).

Table 5. *In vitro* efficacy of Field Formulations and their combinations with effective yeasts against mycelial growth of *Alternaria brassicicola*

Treatment No.	Treatment detail	Per cent mycelial growth inhibition
T1	BK 5 (10%)	94.07 (75.88)*
T2	BK 7 (10%)	84.82 (67.08)
T3	Field Formulation 1 (in water) (10%)	47.78 (43.71)
T4	Field Formulation 2 (with cow urine) (10%)	51.11 (45.62)
T5	Butter milk (5%)	92.96 (74.63)
T6	Field Formulation 1 (5%) + BK 5 (5%)	82.96 (65.60)
T7	Field Formulation 1 (5%) + BK 7 (5%)	52.04 (46.15)
T8	Field Formulation 1(5%) + Butter milk (5%)	78.15 (62.19)
T9	Field Formulation 2 (5%) + BK 5 (5%)	88.52 (70.17)
T10	Field Formulation 2 (5%) + BK 7 (5%)	88.52 (70.20)
T11	Field Formulation 2 (5%) + Butter milk (5%)	84.07 (66.46)
T12	Mancozeb (500ppm)	94.07 (75.89)
T13	Copper oxychloride (500ppm)	80.74 (63.96)
T14	Control	0.00 (0.00)
	$CD_{0.05}$	2.56
	SE(m)	0.88
	SE(d)	1.24

*Figures in parenthesis are arc sign transformed values

Himankshi (2011) reported that five bacterial isolates i.e. LBT3 and LBS2-3 (*Lactococcus*), LBS2-5 and LBS2-7 (*Lactobacillus*) and BS2-4 (*Bacillus*) and only one yeast isolate i.e. YS1-2 (*Saccharomyces* sp.) exhibited antifungal activity. This antagonistic activity has been attributed probably due to secretion of compounds like 3,6-bis (2-methylpropyl)-2 and 5-piperazinedion many metabolites and fungicides from *Bacillus* species against *Sclerotium*, *Alternaria* and *Fusarium* species (Yang and Chang, 2010). Other researchers have also supported these observations (Valdes-Stauber *et al.,* 1991; Ryser *et al.,* 1994; Feio *et al.,* 2004). Barman *et al.* (2018) reported efficacy of two strains of *Lactococcus lactis* subsp. *hordniae* and *Lactococcus lactis* subsp. *lactis* isolated from butter milk against *Alternaria* species. Fungicides were taken as treatments for comparison of efficacy and as found in the present studies the efficacy of fungicide mancozeb in inhibiting the mycelial growth of *Alternaria* spp. is well reported in literature by many workers (Amaresh, 1997; Babu *et al.,* 2001; Prasad and Naik, 2003; Kamal *et al.,* 2007; Sadana and Didwania, 2015).

The results are in agreement with Hossain and Mian, 2005b; Mishra *et al.,* 2009; Gaikwad, 2013; Biswas and Ghosh, 2018 and Valvi *et al.,* 2019. Hossain and Mian (2005b) reported that mancozeb, carbendazim and propiconazole inhibited the mycelial growth of the *A. brassicicola* infecting cabbage. Mishra *et al.* (2009) reported that propiconazole was the most effective against *A. brassicae* which showed maximum per cent inhibition of mycelial growth and was followed by mancozeb. Gaikwad (2013) also reported that propiconazole, difenconazole and mancozeb at different concentrations completely inhibited the mycelial growth of *A. brassicae*. *In vitro* evaluation of fungicide mancozeb 75% WP @ 0.2% against *A. brassicae* revealed maximum reduction in growth and sporulation of fungus at 0.02% concentration (Biswas and Ghosh, 2018). Valvi *et al.* (2019) revealed that, among the different fungicides tested under *in vitro* mancozeb 75% WP (0.25 %) completely inhibited the growth of *Alternaria brassicae* followed by propiconazole 25% EC (0.1%) which showed 96.29 per cent inhibition over control and was statistically at par with mancozeb. No sporulation was observed in mancozeb 75% WP (0.25%), propiconazole 25% EC (0.1%) and difenconazole 25% EC (0.1%).

4.4.2 *In vivo* evaluation

A) Evaluation of botanical formulations, yeasts and fungicides against Alternaria leaf spot of cauliflower under field conditions

The data on per cent disease incidence, per cent disease severity, leaf length and yield revealed that application of each treatment, were taken and mean of which are being presented in Table 6.

BK 5

Mancozeb

Butter milk

Comparative growth of the pathogen in control

Field forumulation 2 + BK 7

Field forumulation 2 + BK 5

BK 7

Comparative growth of the pathogen in control

Plate 7A. ***In vitro*** **efficacy of Field Formulations and their combinations with effective yeasts in causing mycelial inhibition of** ***Alternaria brassicicola***

Field Formulation 2+ Butter milk

Copper oxychloride

Field Formulation 1+ BK 5

Field Formulation1+ Butter milk

Field Formulation 1+ BK 7

Field Formulation 2 (Cow urine)

Field Formulation 1 (Water)

Control

Plate 7B. *In vitro* efficacy of Field Formulations and their combinations with effective yeasts in causing mycelial inhibition of *Alternaria brassicicola*

Table 6. Comparative efficacy of botanical formulations, yeasts and fungicides against Alternaria leaf spot of cauliflower under field conditions

Treatment	Disease incidence (%)	Disease severity (%)	Leaf Length(cm)	Yield (q/ha)
Effective Field formulation 2 (5%)	28 (5.21)**	1.25 (1.48)*	37.28	153.21
Effective Field Formulation 2 (5%) + BK 7 (5%)	12 (3.15)	0.67 (1.27)	36.10	138.60
Effective Field Formulation 2 (5%) + BK 5 (5%)	08 (2.64)	0.42 (1.18)	36.76	176.67
Effective Field Formulation 2 (5%) + Butter milk (5%)	20 (4.53)	1.00 (1.40)	36.72	158.14
Mancozeb (0.25%)	10 (3.11)	0.42 (1.18)	36.72	138.09
Copper oxychloride (0.3%)	34 (5.79)	2.58 (1.84)	34.92	144.14
Butter milk (5%)	10 (4.08)	0.83 (1.34)	36.6	167.33
Control	52 (7.19)	4.08 (2.23)	34.00	123.24
$CD_{0.05}$	1.81	0.39	1.79	27.83
SE(m)	0.62	0.13	0.62	9.56
SE(d)	0.88	0.19	1.13	13.52

*Figures in parenthesis are square root transformed values
**Figures in parenthesis are arc sign transformed values

Five foliar spray of effective Field formulation 2 (5%) + Yeast BK 5 (5%) were given at 10 days interval, started with the initiation of disease proved most efficacious in limiting the Alternaria leaf spot of cauliflower with minimum average disease severity and incidence (0.42 per cent and 8 per cent) and also significantly increased the yield with 176.67 q/ha. Butter milk (5%) with 0.83 per cent disease severity, 10 per cent disease incidence and 167.33 q/ha yield; mancozeb (0.25%) with 0.42 per cent disease severity, 10 per cent disease incidence and 138.09 q/ha yield; Field Formulation 2 (5%) + Yeast BK 7 (5%) with 0.67 per cent disease severity, 12 per cent disease incidence and 138.60 q/ha yield; Field Formulation 2 (5%) + butter milk (5%) with 1 per cent disease severity, 20 per cent disease incidence and 158.14 q/ha yield; Field Formulation 2 with 1.25 per cent disease severity, 28 per cent disease incidence and 153.21 q/ha yield. The foliar spray of copper oxychloride @ 0.3% was least effective in controlling the disease with 2.58 per cent disease severity, 34 per cent disease incidence and 144.14 q/ha yield.

In case of Effective Field Formulation 2 (5%) + yeast BK 5 (5%), was highly effective and the result of present findings are in agreement with Himankshi, 2011 who reported that a mixture of bacterial and yeast isolates along with cow urine was found to be more effective in exhibiting antagonistic activity against all selected phytopathogens (*Alternaria brassicae*, *Colletotrichum truncatum*, *Sclerotium rolfsii*, *Fusarium oxysporum*, *Rhizoctonia solani*) and this may be due to the combined effect of antagonistic compounds secreted by probiotics and volatile and non-volatile compounds of cow urine which probably augmented this anti-microbial activity. The cow urine based botanical extracts along with yeast inhibited the mycelia growth of the pathogen in comparison to control. *Saccharomyces cerevisiae* acts as antimicrobial agent and releases antimicrobial compounds such as antifungal killer toxins (mycocins) (Rima *et al.,* 2012). Many natural plant extracts can inhibit the growth and development of pathogenic fungi, such as methyl thujate, hinokitiol, and cinnamic acid. Li *et al.* (2019) reported that cinnamic acid improved the biocontrol efficacy of *C. laurentii*, which indicates the potential of combined application of natural plant extracts with antagonistic yeasts for controlling postharvest pathogens.

These results of present findings are in agreement with Narain *et al.* (2006) reported that mancozeb was the most effective fungicide against *A. brassicae*. Gaikwad (2013) showed that mancozeb showed the least diseases incidence (15.30%) followed by propiconazole (18.36%) and copper oxychloride (20.65%). Biswas and Ghosh (2018) reported that foliar spray with fungicide mancozeb 75% WP @ 0.2% was found to be most effective in reducing disease severity (81.23%) and infection rate which increased the yield (77.23per cent) of mustard over untreated control. Valvi *et al.* (2019) also reported mancozeb 75% WP as most effective in reducing the disease incidence (28 per cent) of Alternaria leaf spot of cauliflower under field conditions followed by azoxystrobin 23% EC which recorded 29.00 per cent disease incidence.

Sharma (2006) reported that amino acid proline has been found in butter milk which induce resistance in plants and stimulates production of antimicrobial properties. It produces anti-bacterial and anti-fungal metabolites such as phenyl lactic acid, cyclic dipeptides, 3 -hydroxylated fatty acid and several proteinaceous compounds (Garg *et al.,* 2020). Barman *et al.* (2018) reported that under field conditions LABW4 showed good efficacy to control leaf

A. General view of the field experiment

B. Efficacy of Field Formulation 2 + BK 5 isolate of *S. cerevisiae* against Alternaria leaf spot of cauliflower

Plate 8. Field efficacy trial of different treatments and treatment combinations against Alternaria leaf spot of cauliflower

blight disease of Ocimum plant caused by *Alternaria* sp. In greenhouse conditions even in the presence of pathogenic conidial concentration of 1.6×10^4, which was treated only with LABW4 showed that almost all the leaves remained fresh without any disease symptoms.

4.5 MICROBIAL COUNT OF BIO-FORMULATIONS

Serial Dilution Technique was followed for evaluation of microbial count from liquid samples which were used as treatments. Three different media viz. Potato Dextrose Agar Medium (PDA), Yeast Peptone Dextrose Agar Medium (YPDA) and Nutrient Agar Medium (NA) were used for isolation of fungal, actinomycetal and bacterial bioagents, respectively.

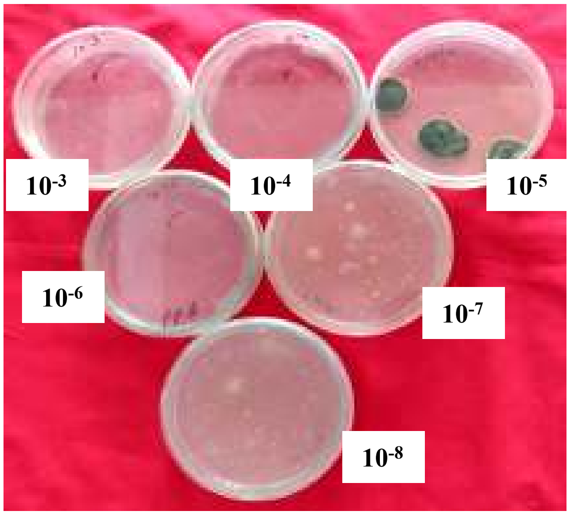
Table 7. Enumeration of microflora count of the treatments

Treatments / Concentrations	FF 2	BM	BK5	BK7	FF 2+ BM	FF 2+ BK5	FF 2+ BK7	Mean A
10^{-3}	0.00	6.75	6.84	7.55	6.18	6.93	7.58	5.98
10^{-4}	0.00	7.21	7.69	8.53	6.58	7.86	8.52	6.63
10^{-5}	6.48	0.00	8.66	8.61	7.31	8.34	8.82	6.89
10^{-6}	0.00	0.00	8.96	9.19	7.93	8.54	9.19	6.26
10^{-7}	9.92	10.29	0.00	0.00	9.93	10.18	10.10	7.20
10^{-8}	10.9	11.26	0.00	0.00	10.62	10.79	10.77	7.77
Mean B	4.56	5.92	5.36	5.65	8.09	8.77	9.16	

Factors	C.D.	SE(d)	SE(m)
Concentration	0.06	0.03	0.02
Treatment	0.07	0.03	0.02
Concentration × Treatment	0.17	0.08	0.06

Out of the seven bio-formulations evaluated under *in vitro* conditions for the microbial count, butter milk had more (log10 (CFU/cm^2) = 11.26) microbial count followed by Field formulation 2 + yeast BK5 (log10 (CFU/cm^2) = 10.77) as compared to other bio-formulations (Table 7).

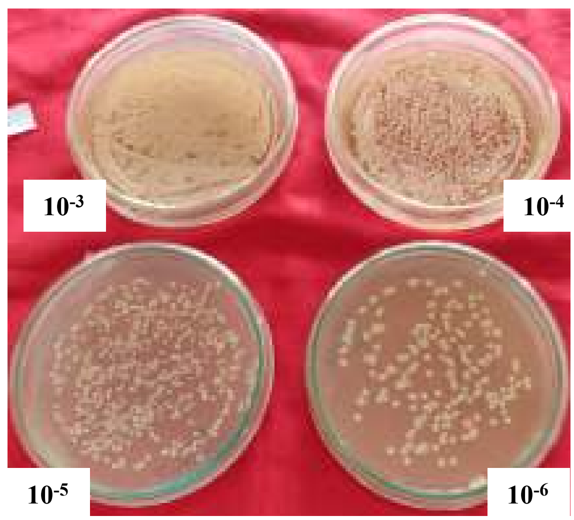
Therefore, results obtained in the present study are in concurrence with the existing literature as given by Shukla *et al.,* 2010; Himankshi, 2011. Fokkema *et al.* (1979) suggested that at least 10^4 CFU/cm^2 of yeast was necessary to control necrotrophic fungal pathogens on rye and wheat leaves and the higher values of antagonist populations might be required to obtain a better control of decay.



A. Field formulation 2

B. Butter milk

C. BK 5

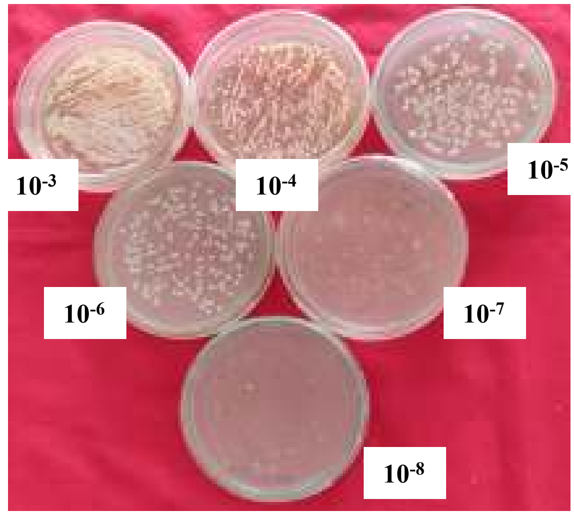


D. BK 7

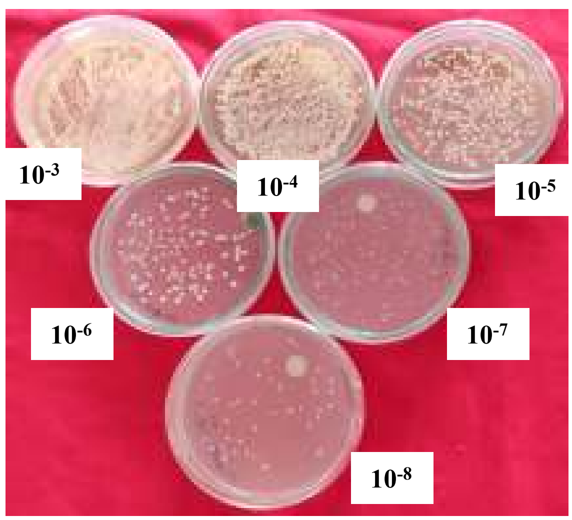
Plate 9. Microflora count of the different treatments

Continuing

E. Field Formulation 2 + Butter milk



F. Field Formulation 2 + BK 5



G. Field Formulation 2 + BK 7

Plate 9. Microflora count of the different treatments

Chapter-5

SUMMARY AND CONCLUSION

The present study on **"Development of bio-formulation based on botanicals and yeasts against *Alternaria* spp. causing leaf spots in cauliflower"** was done to record the occurrence of Alternaria leaf spots of cauliflower. Isolated and identified the associated pathogen through morphological analysis, pathogenicity, physiological studies and evaluated botanicals, yeasts and their combinations for the management of Alternaria leaf spots.

The characteristic disease symptoms of Alternaria leaf spot on leaves were characterized by the presence of yellow, dark brown to black circular leaf spots with target like concentric rings. Under warm and humid weather conditions, centers of the lesions may fall out, giving the leaf spots a shot-hole appearance. Individual spots coalesce into large necrotic areas and leaf drop can occur.

The pure culture obtained from the diseased specimen of leaves was identified as *Alternaria brassicicola* (Schwein.) Wiltshire on the basis of macroscopic and microscopic characters of the test pathogen. Pure cultures of different isolates of yeasts used in this study were also identified on the basis of cultural and morphological characters. All the yeasts were identified as *Saccharomyces cerevisiae* Meyen ex E.C. Hansen. The cultural study indicated that the yeasts produced white; creamy pigmentation, oblong/ eclipse colony with cell length of 2-8 μm and cell breadth of 2-5 μm and attained a full growth of 90 mm on Yeast Peptone Dextrose Agar medium at 25±1 ^{0}C in 3-4 days.

Pathogenicity of *Alternaria brassicicola* was proved following Koch's postulates on cauliflower cv. "Pusa Snowball K1" on one month old seedlings in the pots and also through detached leaf technique. Initial symptoms on the leaves of the potted plant appeared after 120.3 hours of inoculation as small, isolated scattered pale yellow to brown spot with a yellow halo. On the detached leaves, the initial symptoms of the disease appeared as small yellow fleck with light brown centre after 62.1 hours of inoculation. In advanced stages, the necrotic spots enlarged in size covering the whole leaf lamina giving them blighted appearance.

While studying the morphology it was found that in the initial stages, the colony colour was dull white in appearance which later turned to olivaceous brown to greenish black in colour. Hyphae of *Alternaria brassicicola* was septate and profusely branched with a diameter ranging from 2.5-6.02 µm and average diameter of 4.26 µm. Conidia of the pathogen were dark, cylindrical to oblong in shape and are muriform with no beak formation. Size of conidia ranged from 44-55 × 11-16 µm with average size of 49.5 × 13.5 µm. Generally, conidia had 5-8 transverse and 0-5 longitudinal septa, respectively.

Out of nine botanicals evaluated under *in vitro* conditions against Alternaria leaf spot pathogen, leaf extract of Karvaya (*Roylea elegans*) was found most effective and significantly superior amongst all the treatments with 52.64 per cent average inhibition in mycelial growth of the pathogen at 10 per cent concentration followed by Neem and Aloe with 51.11 per cent and 46.67 per cent mycelia inhibition, respectively.

Out of six yeasts isolates evaluated under *in vitro* conditions against *Alternaria brassicicola*, BK 5 and BK 7 were found most effective and significantly superior with 59.72 and 55.56 per cent average inhibition in mycelial growth of the pathogen, respectively. BK 1 (43.61 per cent) was found to be the least inhibitory.

Two Field Formulations (1 and 2) of the five effective botanicals, two effective yeasts, buttermilk and their combinations were evaluated under *in vitro* conditions and Yeast BK5 was found most effective with 94.07 per cent average inhibition in mycelial growth of the pathogen which was comparable with mancozeb. Butter milk was found next in efficacy with 92.96 per cent mycelia inhibition.

Five foliar spray of effective Field formulation 2 (5%) + Yeast BK 5 (5%) were given at 10 days interval, started with the initiation of disease proved most efficacious in limiting the Alternaria leaf spot of cauliflower with minimum average disease severity and incidence (0.42 per cent and 8 per cent) and also significantly increased the yield with 176.67 q/ha. Butter milk (5%) with 0.83 per cent disease severity, 10 per cent disease incidence and 167.33 q/ha yield; mancozeb (0.25%) with 0.42 per cent disease severity, 10 per cent disease incidence and 138.09 q/ha yield; Field Formulation 2 (5%) + Yeast BK 7 (5%) with 0.67 per cent disease severity, 12 per cent disease incidence and 138.60 q/ha yield; Field Formulation 2 (5%) + butter milk (5%) with 1 per cent disease severity, 20 per cent disease incidence and

158.14 q/ha yield; Field Formulation 2 with 1.25 per cent disease severity, 28 per cent disease incidence and 153.21 q/ha yield. The foliar spray of copper oxychloride @ 0.3% was least effective in controlling the disease with 2.58 per cent disease severity, 34 per cent disease incidence and 144.14 q/ha yield.

Microbial count of the yeast based foliar sprays was also determined to find out the microbial strength of the treatments. Butter milk had more ($\log_{10}$ (CFU/cm^2) = 11.26) microbial count followed by Field formulation 2 + Yeast BK 5 (10.77) as compared to other bio-formulations.

CONCLUSION:

On the basis of the investigations done in the present study, *Alternaria brassicicola* was found the incitant of Alternaria leaf spot disease of cauliflower with small yellow fleck with light brown centre on the leaves which further turned into yellow to brown dark spots with a yellow halo which further increased in size and became black necrotic circular to irregular in form. In advanced stages, the necrotic spots enlarged in size covering the whole leaf lamina giving them blighted appearance. Five foliar application of Field Formulation 2 (5%) + Yeast BK 5 (5%) and Butter milk at 10 days interval starting from first spray with the onset of disease symptoms till the harvest of the crop proved as most effective in reducing the disease incidence (8 per cent and 10 per cent), disease severity (0.42 per cent and 0.83 per cent) and enhancing the crop yield (176.67 q/ha and 167.33 q/ha), respectively.

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APPENDIX I

Culture media used for growing of microorganisms

1. Potato dextrose agar medium

Agar-agar	20.00 g
Dextrose	20.00 g
Peeled potato	250 g
Distilled water	1000 ml

2. Yeast Peptone Dextrose Agar Medium

Yeast extract	5 g
Peptone	5 g
Dextrose	10 g
Agar-agar	20 g
Distilled water	1000 ml
pH	6.5

3. Nutrient Agar Medium

Beef extract	1.5 g
Yeast extract	1.5 g
Peptic digest animal tissue	5 g
Sodium chloride	5 g
Agar-agar	15 g
Distilled water	1000 ml
pH	7.4 ±

APPENDIX II

ANOVA TABLES

ANOVA 1: *In vitro* efficacy of different botanicals at 10 per cent concentration against mycelial inhibition of *Alternaria brassicicola* causing leaf spot of cauliflower (Table 3)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Signficance
Treatment	9	6,486.26	720.695	838.034	0.00000
Error	30	25.799	0.86		
Total	39	6,512.05			

ANOVA 2: *In vitro* efficacy of different isolates of yeasts against mycelial growth of *Alternaria brassicicola* (Table 4)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Signficance
Treatment	6	7,747.93	1,291.32	999.26	0.00000
Error	21	27.138	1.292		
Total	27	7,775.07			

ANOVA 3: *In vitro* efficacy of Field Formulations and their combinations with effective yeasts against mycelial growth of *Alternaria brassicicola* (Table 5)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Signficance
Treatment	13	15,968.72	1,228.36	529.618	0.00000
Error	28	64.942	2.319		
Total	41	16,033.66			

ANOVA 4: Comparative efficacy of botanical formulations, yeasts and fungicides on yield against Alternaria leaf spot of cauliflower under field conditions (Table 6)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Signficance
Replication	4	764.866			
Treatment	7	10,553.37	1,507.62	3.301	0.01094
Error	28	12,789.14	456.755		
Total	39	24,107.37			

ANOVA 5: Comparative efficacy of botanical formulations, yeasts and fungicides on leaf length against Alternaria leaf spot of cauliflower under field conditions (Table 6)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Signficance
Replication	4	8.164			
Treatment	7	43.18	6.169	3.258	0.0117
Error	28	53.022	1.894		
Total	39	104.365			

ANOVA 6: Comparative efficacy of botanical formulations, yeasts and fungicides on disease severity against Alternaria leaf spot of cauliflower under field conditions (Table 6)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Signficance
Replication	4	0.268			
Treatment	7	4.736	0.677	7.636	0.00004
Error	28	2.481	0.089		
Total	39	7.484			

ANOVA 7: Comparative efficacy of botanical formulations, yeasts and fungicides on disease incidence against Alternaria leaf spot of cauliflower under field conditions (Table 6)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Signficance
Replication	4	5.75			
Treatment	7	83.965	11.995	6.196	0.00019
Error	28	54.207	1.936		
Total	39	143.923			

ANOVA 8: Enumeration of microflora count of the treatments (Table 7)

Source of Variation	DF	Sum of Squares	Mean Squares	F-Calculated	Signficance
Factor A	5	44.192	8.838	845.654	-0.00000
Factor B	6	366.593	61.099	5,845.911	0.00000
Intraction A X B	30	1,379.961	45.999	4,401.141	-0.00000
Error	84	0.878	0.010		
Total	125	1,791.624			

Department of Plant Pathology
Dr Yashwant Singh Parmar University of Horticulture and Forestry
Nauni, Solan (HP) 173 230

Title of Thesis	:	**“Development of bio-formulation based on botanicals and yeasts against *Alternaria* spp. causing leaf spots in cauliflower”**
Name of the Student	:	Sonali Parwan
Admission Number	:	H-2019-73-M
Major Advisor	:	Dr. Harender Raj Gautam
Major Discipline	:	Plant Pathology
Minor Discipline	:	Vegetable
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ABSTRACT

The present study on Alternaria leaf spot of cauliflower (*Alternaria brassicicola* (Schwein.) Wiltshire) were undertaken in relation to prevalence during 2019 and 2020 crop seasons. Characteristic symptoms of the disease were characterized by the presence of yellow, dark brown to black circular leaf spots with target like concentric rings. Under warm and humid weather conditions, centers of the lesions may fall out, giving the leaf spots a shot-hole appearance. Individual spots coalesce into large necrotic areas and leaf drop can occur. The pure culture of the pathogen was isolated over PDA. While studying the morphology of the test pathogen it was found that the mycelia (2.5-6.02 µm) were brown or olivaceous brown, septate and profusely branched in old cultures whereas pathogen produced conidiophores of 35-45×5-8 µm on which dark conidia were formed which ranged from 44-55×11-16 µm in size and had 5-8 transverse and 0-5 longitudinal septa and are muriform with no beak formation. On the basis of morphological characters, the associated pathogen was identified as *Alternaria brassicicola* (Schwein.) Wiltshire. Pathogenicity of *Alternaria brassicicola* was proved following Koch’s postulates on cauliflower cv. “Pusa Snowball K1”. The pathogen exhibited initial symptoms after 120.30 h and 62.10 h of inoculation on potted plants and detached leaves, respectively. For disease management, botanicals, yeasts and their combinations were evaluated against test pathogen both under *in vitro* and *in vivo* conditions. Among botanicals, Karvaya (*Roylea elegans*) (52.64 per cent) recorded maximum mycelial inhibition while in case of yeasts, BK5 and BK7 was most effective and significantly superior to all other treatments with 59.72 and 55.56 per cent inhibition in mycelial growth, respectively. Out of botanicals, yeasts and well known fungicides, Yeast BK5 @ 5 per cent was found superior with 94.07 per cent mycelial growth inhibition which was comparable with Mancozeb @ 0.25 per cent. Microbial count of the yeast based foliar sprays was determined to find out the microbial strength of the treatments. Butter milk had more (log10 (CFU/cm^2) = 11.26) microbial count followed by Field formulation 2 + Yeast BK5 (10.77) as compared to other bio-formulations. Field evaluation studies referred the application of five foliar sprays of Field formulation 2 + Yeast BK5 @ 5 per cent at 10 day interval started with the inhibition of disease as most efficacious in limiting the Alternaria leaf spot of cauliflower (8 per cent) and enhancing the yield (176.67 q/ha).

Signature of the Student	**Signature of the Major Advisor**
Name: Sonali Parwan	**Name:** Dr. Harender Raj Gautam
Date:	**Date:**

Countersigned

Professor and Head
Department of Plant Pathology
Dr Yashwant Singh Parmar University of Horticulture and Forestry
Nauni, Solan, (HP) - 173 230

BRIEF BIO-DATA

Name	**:**	**Sonali Parwan**
Father's Name	**:**	Shri Parvesh Parwan
Mother'Name	**:**	Smt. Meenu
Date of Birth	**:**	16.10.1995
Sex	**:**	Female
Marital status	**:**	Unmarried
Nationality	**:**	Indian

Academic Qualification:

Examination Passed	Year of Passing	University/Board	Division	OCPA/OGPA (% equivalent)
Matriculation	2012	ICSE	FIRST	82.80%
10+2	2014	CBSE	FIRST	79.20%
Graduation	2019	CSK HPKV, Palampur (H.P)	FIRST	75.00%

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(Sonali Parwan)