MOLECULAR CHARACTERIZATION OF *Alternaria alternata* CAUSING FRUIT ROT OF CHILLI THROUGH RAPD MARKER

THESIS

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By

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DECLARATION OF STUDENT

I hereby declare that the experimental work and its interpretation of the Thesis entitled **"MOLECULAR CHARACTERIZATION OF *Alternaria alternata* CAUSING FRUIT ROT OF CHILLI THROUGH RAPD MARKER"** or part thereof has neither been submitted for any other degree or diploma of any University, nor the data have been derived from any thesis / publication of any University or scientific organization. The source of material used and all assistance received during the course of investigation have been duly acknowledged.

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CERTIFICATE

This is to certify that thesis entitled **"MOLECULAR CHARACTERIZATION OF *Alternaria alternata* CAUSING FRUIT ROT OF CHILLI THROUGH RAPD MARKER "** submitted in partial fulfillment of the requirement for the degree of **"Master of Science in Agriculture (Plant Pathology)"** of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola is a record of bonafide research work carried out by **Jankar kajal Dadasaheb** under my guidance and supervision.

The subject of the thesis has been approved by the Student's Advisory Committee.

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(D) Abbreviations

%	Per cent
/	per
@	At the rate
<	Lesser than
µ	Micron
µm	Micrometer
^{0}C	Degree Centigrade
Agril.	Agricultural
BOD	Biological oxygen demand
Bp	Base Pair
CTAB	Cetyl Trimethyl Ammonium Bromide
CDA	Czapek's Dox Agar
CMA	Corn meal Agar
DAI	Days after inoculation
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphates
Dr. PDKV, Akola	Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola
e.g.	For example
EDTA Na_2	Ethylene Diamine Tetraacetic Acid DiSodium
et al.	et alia (and others)
etc.	Etcetera
Fig.	Figure
Hr.	Hour (s)
i.e.	That is
ITS	Internal transcript Spacer
Mbp	Million Base Pair
$MgCl_2$	Magnesium chloride
ml	Milliliter (s)

mm	Millimeter
mM	Mili Molar
NaCI	Sodium Chloride
No.	Number
OMA	Oat Meal Agar
PCR	Polymerase Chain Reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
RAPD	Randomly amplified polymorphic DNA
Sr. No.	Serial number
Taq	*Thermus aquaticus*
TBE	Tris-borate-EDTA
U/µl	Unit per micro liter
UPGMA	Unweighed Pair Group Method with Arithmetic Mean
UV	Ultra Violet
Viz.	Videlicet namely

E) THESIS ABSTRACT

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ABSTRACT

Fruit rot of chilli caused by *Alternaria alternata* is one of the major constraint in total production of chilli. In present investigation various aspects studied regarding variability of *A. alternata.* Six isolates of *A. alternata* selected for morphological variability studies of different chilli

growing districts of Maharashtra. Isolates showed variation in morphology *viz*., radial growth, colony colour, colony margin, size of conidia, shape and septation of conidia and sporulation on five different media i.e PDA, OMA, CDA, V-8 and CMA. Radial growth ranging 75.65 to 31.66 mm, conidia were muriform, broadly rounded base to obclavate, oval in shape. Conidial size was in the range of 43.30-27.7×12.4-8.3 µm on different media. Trasverse and longitudinal septation of conidia 7-9 to 3-4, 0-3 to 0-1, respectively. Colour of colony were greenish to greenish brown, grayish black, creamy white with regular to irregular margin on different media. Good sporulation i.e 2.8 was noticed in PDA media.

The ripe chilli fruit inoculated with *A. Alternata.* All the isolates are pathogenic, while Isolate Aa5 (Nagpur) was the most virulent isolate and Aa4 (Akola) was the less virulent isolate.

The molecular variability was studied amongst the 6 isolates of *A. alternata* by using 15 RAPD primers of OPA and OPB series off which 10 primers produced 83 scorable bands. Among the RAPD primer 83 bands 80 bands were polymorphic and level of polymorphism was 96.38%. Molecular diversity using RAPD marker showed that the Aa2 (Satara) having higher similarity index with Aa4 (Akola).

CHAPTER I

INTRODUCTION

1.1 Background Information

Chilli (*Capsicum annuum* L.) is one of the most important commercial spice and export crop originated from Tropical America. It is grown throughout the world for its green and red ripe fruit. India is the largest grower, consumer and exporter of chilli, currently exporting 3.47 lakh tonnes dry chilli and chilli products over 90 countries around the world. In India the area under green chilli cultivation 287’000 ha with production 3407 MT and productivity 12 MT/ha. While, in case of dried chilli 837’000 ha area is under cultivation with 1872 MT production and 2 MT/ha productivity (NHB, 2016-17) comprising Andhra Pradesh, Karnataka, Maharashtra, Orissa, Tamil nadu, Bihar, Uttar Pradesh are the major chilli growing states of India. In Maharashtra the total area under green chilli cultivation is 12.29’000 ha with production 127.41MT (NHB, 2014-15).

Chilli fruit is used as fresh, cooked, pickled, canned in sauce and powder in hot spices. Green chillies are rich source of vitamins especially vitamin A, C, B_1, B_2. Chilli belongs to solanaceae family having 20-27 species, of which five *viz*., *C. annuum*, *C. baccatum*, *C. Chinese*, *C. frutescens* and *C. pubescens* are domesticated in different parts of the world.

Chilli forms an indispensable adjunct essentially used in every Indian cuisine due to its pungency, spicy taste, appealing odour and flavour. It is used as vegetable in several preparations. Chilli extracts are used in a wide range of medicines against tonsillitis, diphtheria, loss of appetite, flatulence, intermittent fever, rheumatism, sore throat, swellings and hardened tumors.

The sustainability of chilli based agriculture is threatened by a number of factors. The biotic stresses such as bacterial, fungal, viruses and several insect pest damage and abiotic factor have been reported to impair the crop productivity. Anthracnose, fruit rot, die back, bacterial wilt and leaf curl causes loss in chilli. Chilli fruit rot primarily incited by *Alternaria*

alternata is one of the major economic constraints to chilli production worldwide. *A*. *alternata* belongs to kingdom fungi, phylum Ascomycota, class Dothideomycetes, subclass Pleosporomycetidae, order Pleosporales, family Pleasporaceae and Genus *Alternaria*. *Alternaria* species are widely distributed, infecting a broad range of economically important crops. The great majority of *Alternaria* species are saprobic or have been described as occurring on hosts of little economic importance, but some species such as *A*. *alternata* (Fr.) Keissl., *A*. *solani* Sorauer, and *A*. *brassicae* (Berk.) Sacc. are well known as destructive pathogens (Simmons 2007). *A*. *alternata* cause diseases in potato, tomato, cotton, amaranthus, watermelon, etc.

In India, the first report of *Alternaria* sp. was made from Delhi by Dutt in 1937. Mathur and Agnihotri (1961) reported fruit rot of chilli caused by *Alternaria tenuis* Nees from Rajasthan reported 5-85 per cent yield losses due to this disease. Sreekantiah et al.(1973) reported a virulent strain of *A. alternata* causing leaf spot and fruit rot of chilli from Mysore, while from Maharashtra, Khodke and Gahukar (1993) also reported *Alternaria* sp. associated with chilli. Bhatt et al. (2000) recorded *A. alternata* causing fruit rot on chilli from Kumaon hillis of Uttar Pradesh, India. Narain et al. (2000) reported *Alternaria alternata* causing fruit rot of chilli on fruits, as initially small blackish brown, circular to elongated water soaked depressed lesions are formed on the pericarp of fruits, which leads to rotting of fruits in later stage. The characteristic lesions observed at semi ripe stage of chilli fruits. Fruit rot is a major constraint in chilli causing several losses in terms of quality and quantity.

Correct and accurate identification leads to more effective disease control and management. In addition to morphological variation genetic variation of plant pathogen may be of great importance to minimize disease incidence. Breeding for disease resistance plant with durable resistance requires resistance against all pathotype of the pathogen. Highly variable plant pathogen may be difficult to combat the development of resistant plant genotype. The DNA based techniques called Random Amplified Polymorphic DNA(RAPD) constitutes one of the method for discriminating among strain of species and is often deemed superior in

resolution or efficacy to traditional traits. The use of resistant varieties not only eliminated losses from diseases, but also eliminated chemical and mechanical expenses of disease control.

1.2 Importance of study

Chilli has commercial importance as a cash and spice crop. It has many advantages. It comprises numerous chemicals including steam volatile oils, fatty oils, capsaicinoid, carotenoids, vitamins, proteins, fibre and mineral elements. Many chilli constituents are important for nutritional value, flavor, aroma, texture and colour. Chillies are low in sodium and cholesterol free rich in vitamin A and C and are a good source of potassium, folic acid and vitamin E. fresh green chilli contain more vitamin C than citrus fruit and red chilli contain more vitamin A than carrot.

Two chemical groups produced by chilli capsaicinoid and carotenoids. The capsaicinoid are alkaloids that make hot chilli pungent. A large number of carotenoid provides high nutritional value and the colour to chilli.

Fruit rot is the key disease of chilli production and rank first among fungal diseases and affecting both fruit and seed quality. Virulence and pathological variability within the pathogen due to geographical and environmental situation also plays an important role in disease development. Mathur and Agnihotri (1961) and Singh (1987) reported 5-85 per cent yield losses due to this disease.

To overcome the traditional morphology based identification schemes, DNA sequence analyses have been used to characterize and analyze the taxonomic complexity of *Alternaria* spp. Characterization of diverse populations of *A*. *alternata* could improve the knowledge of population biology and its interaction to contribute to the design of optimal breeding methods to produce chilli cultivars with stable and durable resistance to fruit rot.

1.3 Objectives of study

Keeping in view, the importance of this crop and potential threat of fruit rot in almost all the chilli growing areas in the world, it was

proposed to study the variability of *A*. *alternata* isolates causing fruit rot in chilli collected from different chilli-growing regions of Maharashtra on their morphological and molecular level. Therefore, the Present investigation was undertaken with following objectives:

1. To study the morphological and molecular variability of *Alternaria alternata* causing fruit rot of chilli through RAPD marker.

1.4 Scope and limitation

The knowledge on the presence of different race types/pathotypes of *Alternaria alternata*, population which is important to control the fruit rot disease. The correct and accurate taxonomic identification is necessary for plant breeding purposes and disease management. Traditionally identification and characterization of *Alternaria* species was based on morphological characters such as size and shape of conidia and cultural characters such as colony colour, growth rate and texture. These criteria alone are not always adequate to differentiate species because of variations in morphology and phenotype among species under different environmental conditions. In recent years, Polymerase Chain Reaction (PCR) based methods have emerged as major tools for the diagnosis of plant diseases. Therefore to overcome taxonomic problems associated with these traditional identification methods, pathological variation among the isolates of *Alternaria alternata* and DNA sequence analysis were used to study the genetical variation among the isolates.

Hence, it would be necessary to ascertain the genetic and pathogenic variability amongst the pathogen. In view of the above, an investigation was carried out on “Molecular characterization of *Alternaria alternata* causing fruit rot of chilli through RAPD marker.*”* during 2015-2017 at Department of Plant Pathology, Post Graduate Institute, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

1.5 Hypothesis or assumption

The isolates of *A. alternata* collected from different geographical situation may be helpful for understanding the pathogenic nature existed within the isolates of *A. alternata* for management of disease. The variability of *A*. *alternata* population in chilli growing area pose the difficulties in developing resistance in chilli.

The genetical variation among the isolates of *A. alternata* may also be detected with the help of molecular technique RAPD using specific primers. These studies will showed the genetical variability among the isolates of *A. alternata* collected from different geographical area and to know the prevalence and distribution of more virulent isolates of fruit rot pathogens in the country. The genetic variation among the isolates will be used for screening of breeding material and deplay varieties that resist to many isolates.

CHAPTER II

REVIEW OF LITERATURE

Studies related to the *Alternaria alternata* causing fruit rot in chilli was undertaken and attempts have been made to put forth the recently published worked reviewed critically under the following major heads.

2.1 History

Droby et al. (1984) reported *Alternaria alternata* (Fr.) Keissler causing brown spot of potato in Israel.

Khodke and Gahukar (1993) also reported *Alternaria alternata* (Fr.) Keissler from Maharashtra.

Pryor and Michailides (2002) reported *Alternaria alternata* from late blight of pistachio as one of the most common fungal diseases of pistachio in California, affects both foliage and fruit.

Weber and Halterman (2012) reported early blight of potato, caused by *Alternaria solani* Sorauer, resulted in economic losses in many potato growing regions.

Jarchelou et al. (2013) isolated one fourty one *Alternaria* species from potato fields of West Azerbaijan province. Based on macro and micromorphological characters of the isolates, 9 species *viz*,: *A. alternata*, *A. broussonetiae*, *A. destruens*, *A. dumosa*, *A. interrupta*, *A. rhadina*, *A. solani*, *A. soliaegyptiacae* and *A. tenuissima* were identified. Among the identified species, 5 species: *A. alternata*, *A. dumosa*, *A. interrupta*, *A. solani* and *A. tenuissima* were reported previously from potato, but three species: *A. broussonetiae*, *A. rhadina* and *A. soliaegyptiaca* are new to mycoflora of Iran and are reported for the first time from potato plants.

Dalphy et al. (2014) reported four *Alternaria* species group (*A. longipes, A. arborescens, A. alternata and A. mali*) associated with leaf blotch and fruit spot of apple in Australia having yield losses upto 15-25%.

Nasehi et al. (2014) reported a new species of *Alternaria* causing leaf spot of pepper (*Capsicum annuum*) obtained from the Cameron highlands, Pahang, Malaysia, was determined based on phylogenetic analyses, morphological characteristics, and pathogenicity assays as *Alternaria capsicola.*

2.2 Isolation, Identification and Symptomatology

Pryor and Michailides (2002) isolated *Alternaria* spp. from pistachio tissue collected from five orchards in California during three different period of growing season i.e during spring, summer and in fall. The *Alternaria* isolates were identified based on morphological and molecular characterization. The late blight of pistachio produce typical symptoms as large necrotic lesions, eventually coalesce and consume the entire leaf. Which are black in the center due to the abundant production of fungal spores and surrounded by a chlorotic halo. On fruit, the disease is characterized by the development of small necrotic spots surrounded by a red halo on the hull of immature nuts.

Pusz (2009) isolated *Alternaria alternata* from seeds of *Amaranthus cruentus, A. paniculatus* and *A. retroflexus,* were highly pathogenic to *A. cruentus* and *A. paniculatus* seedlings *in vitro*. The pathogen produced necrotic lesions on stem and leaves, and caused seedling wilt and death.

Khodaei and Arzanlou (2013) recorded total ninty nine *Alternaria* isolates were recorded from infected leaves of sunflower and identified as *A. alternata* based upon morphological characteristics like colony colour, conidiophores and conidia formation. Sympatology of *A. alternata* was studied as pale to dark brown to black, paler at center, irregular in shape surrounded by necrotic halo produced on all over the leaf blade, Later on spots were produced on stems and reproductive organs. In severe infections the spots coalesced resulting in leaf blight and rotting of stem and heads.

Abeer et al. (2014) isolated *Alternaria alternata* (Fr.) Keissler from *A. marina* (forsk) and identified based on morphological

characteristics given by simmons, 2007. *A. alternata* produced symptoms as bright to pale yellow or tan flecks on the upper leaf surface surrounded by yellow halo. In the early stage, these flecks surrounded by greasy (water soaked) appearance while older spots were circular to irregularly lobed and are light brown-black in colour with concentric rings.

Zahra Ibrahim El-Gali (2015) isolated *Alternaria* isolates from carob (*Ceratonia siliqua* L.) from Libya in Mediterranean region. Based on the symptoms development on leaves and identified as *A. alternata* on the basis of their colonial morphology, microscopic examination at vegetative and sporulating stage. The sympatological study showed the formation of small dark circular brown spots on the leaves. Which later on increased in size and become blotch or sunken lesions, these lesions gradually exhibited a grayish tint at the center surrounded by a yellow halo.

Zhao et al. (2016) reported total sixty four *Alternaria* isolates recorded from symptomatic leaf sample of watermelon collected from seven districts in Beijing municipality of China. Among these, fourty nine isolates were identified as *A. tenuissima* and fifteen isolates were confirmed as *A. alternata* based on morphological traits and molecular characteristics.

Devi et al. (2016) studied the sympatology of sunflower leaf showing the typical symptoms of leaf blight collected from 10 sunflower growing area of Tamil nadu. The pathogen was isolated on Potato Dextrose Agar and isolate identified as *A. helianthi* based on morphological characteristics.

Devappa and Thejakumar (2016) isolated *Alternaria* from infected chilli leaves and identified as *A. alternata* based on morphological characterization. Symptoms of Alternaria leaf spot appear on leaves as a small, circular necrotic spot with irregular margin and it remained brown in colour surrounded by yellow halo. Later size and number of spots increased and the spots coalesced.

2.3 Pathogenicity and pathogenic variability

Droby et al. (1984) proved pathogenicity on potato cultivars by inoculating detached leaves or whole plant with conidial suspension of *A. alternata* 10^3, 10^4, 10^5 per ml spore concentration. Typical symptoms consist of brown spots, few millimeter in size, dispersed all over the leaf surface were produced after inoculation of *A. alternata*.

Kumar et al. (2008) proved pathogenicity test of *A*. *solani* conducted in polyhouse conditions using three highly susceptible varieties of tomato i.e. KDTS-71, CO-3 and Punjab Chhuhara of one month old seedling by using culture suspension. Six isolates (So, Dh, Va-5, Hy, Ba-1 and My) were found to be virulent, causing severe disease in all tested varieties, the percent disease incidence of virulent isolates ranged between 73.9 and 83.35%. Other isolates were avirulent because these isolates were unable to cause symptoms of the disease, and avirulent PDI ranged between 0 and 42.07%.

Jadhav et al. (2011) studied the pathogenic variability of *A. macrospora* isolated from cotton growing area of Maharashtra. Total ten isolates were studied for their pathogenic variability by inoculating susceptible cotton variety seedlings LRA-5166 with spore suspension. Among all ten isolates, the isolate I_1, I_2 and I_3 (lesion size1.9 to 2.1 mm) were found highly virulent as they caused infection within 8-9 days.

Sofi et al. (2013) studied pathogenicity of *A*. *mali* causing leaf blotch of apple by inoculating with conidial suspension (4×10^5) on detached leaves. Total twenty one isolates were collected from Kashmir valley. The incubation period of isolates varied from 2 to 6 days. The minimum incubation period was recorded in isolates Am-9, Am-13 and Am-15, and maximum in the isolate Am-17. The number of lesions produced by isolates varied from 6.3 to 14.3 with least in Am-2 and maximum in Am-16. The minimum lesion size of 2.9 mm was observed in Am-17 and maximum of 10.2 mm was seen in Am-1.

Jarchelou et al. (2013) studied pathogenicity of *Alternaria* spp. on potato cultivar. Total 141 isolates belonging to the *Alternaria* were isolated and purified. All the isolates were found pathogenic, based on diameter of necrotic area varied among different species. Among one fourty one isolates *A. tenuissima* had the highest frequency and *A. solani* and *A. tenuissima* had the highest degree of pathogenicity.

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

Singh et al. (2014) studied ten isolates of *Alternaria solani* under polyhouse for pathogenicity test. Among ten isolates, three group were formed on the basis of SAS analysis in which, Group A isolates comprising (MF-4 and PN-4), were highly virulent. Group C indicated virulence (BG, AF-2, EC-1 and RF-1) with mean AUPDC and Group E isolates were less virulent (BHU-1, IIVR, SF-1 and BX-2) with mean AUPDC.

Nasehi et al. (2014) conducted pathogenicity test of *A*. *capsicicola* by spraying 30 day-old pepper plants of Malaysian cultivar BBS010 with a spore suspension 1×10^5 conidia. Symptoms of leaf spot were observed in all of the inoculated pepper plants 14 days after inoculation. The disease incidence was 100.00 % in the inoculated plants, and the disease severity of the leaves was 47.00%. No symptoms were observed in the control plants. The symptoms on the inoculated plants were similar to those observed in the greenhouse.

Dalphy et al. (2014) used four *Alternaria* species groups (*A. longipes, A. arborescens, A. alternata and A. tenuissima*) to prove pathogenicity. Total sixteen isolates (four of each) were used to prove pathogenicity. Among sixteen, twelve isolates showed leaf blotch symptoms while in case of fruit inoculation *A. alternata*, *A. tenuissima*, and *A. longipes* species group showed fruit spot symptoms, whereas *A.*

arborescens did not show fruit rot symptoms. Thus, considerable variation was shown in pathogenicity and virulence among isolates within the species groups.

Zahra Ibrahim El-Gali (2015) conducted pathogenicity test of *Alternaria alternata* on detached healthy leaves of *Ceratonia siliqua*. Five days after inoculation the symptoms first appeared in the form of small dark circular brown spots on the leaves. Seven days after inoculation, these spots increased in size and became blotch or sunken lesions. The lesion gradually exhibited a grayish tint at the centre surrounded by a yellow halo. Ten days after inoculation, the estimated disease severity on leaves of *Ceratonia siliqua* was approximately 80.00%.

Devappa and Thejakumar (2016) proved pathogenicity by inoculating a healthy chilli seedling with spore suspension from 10 day old culture of *A. alternata.* Symptoms of Alternaria leaf spot started appearing from lower side of leaves on seven to nine days after inoculation as a small, circular necrotic spot. These spots started to increase with irregular margin and remained brown in colour surrounded by yellow halo.

Zhao et al. (2016) conducted pathogenicity test on detached leaves from 40-day old plants of watermelon using eight isolates. The inoculated watermelon leaves developed dark brown lesions similar to those initially observed in the field, after 7 days incubation. The disease incidence and disease index of watermelon leaves inoculated with *A. tenuissima* were 61.1-83.3% (72.2±11.1%) and 15.28-29.17 (20.83±7.35) and by *A. alternata* were a little lower, which were 44.4-61.1% (53.7±8.5%) and 16.67-23.61 (21.30±4.01), respectively.

Devi et al. (2016) studied the pathogenicity of *A. helianthi* on the susceptible Sunflower (Vr.CO-3). Ten days old sunflower plants were inoculated with conidial suspension (5×10^5 spores ml^{-1}) of *A. helianthi*. Among the ten isolates, isolate I_3 collected from Naranapuram was the most aggressive (70.22 %) while I_7 collected from Kovilpatti was the least virulent (19.11%).

2.4 Morphological Variation

Pryor and Michailides (2002) studied morphological characteristics of *Alternaria* based on single-spored colonies and grouped into four colony types. Group 1 isolates produced lettuce green to olive green colonies and texture was felty to woolly and did not produce diffusible pigments. The isolates produce colonies over 70 mm in diameter after 7 to 10 days. Group 2 isolates produced pale olive gray to olive gray colonies. Colony texture was woolly to cottony and did not produce diffusible pigments. Isolates typically produced colonies 50 to 70 mm in diameter after 7 to 10 days. Group 3 isolates produced colonies that were typically dark olive gray to iron gray to castor gray in color. Colony texture was felty to woolly. Colony growth was <50 mm produced a diffusible pale orange pigment. Group 4 isolates produced colonies either white to pale gray or apricot orange. Colonies generally had a cottony texture. No diffusible pigments. Isolates typically produced colonies over 70 mm in diameter.

Kumar et al. (2008) isolated eleven isolates of *A. solani* from tomato and designated as So, Dh, Sh, Va-5, Ka, Ma, Hy, Ba-1, My, Va-3 and Mi from different agroclimatic condition. The pigmentation varied from yellow, brown, black, brownish to greenish black in isolates of *A*. *solani* on potato dextrose agar medium. In general, radial growth of all isolates ranged between 14.9 mm and 32.2 mm on PDA and 24.3 mm to 53.7 mm on three selective media i.e., ASM, V-8 juice agar and V-8 juice agar (synthetic) on the fourth day. The fastest radial growth was recorded in the So isolate and slowest in the Ka isolate on PDA, while isolates Dh, Ba-1 and Va-3 were recorded to be faster in growth on ASM, V-8 juice agar and V-8 juice agar (synthetic) medium. The thickness of conidiogenous hyphae varied between 1.17 µ and 9.56 µ, with maximum in the Va-5 and Ma isolates. Most of the isolates showed smooth mycelia growth with circular and irregular margin. Sporulation was not found in all isolates.

Pusz (2009) isolated *A. alternata* from the genus Amaranthus including species grown for food (*A.cruentas*), ornamentals (*A. paniculatus*) as well as weeds (*A. retroflexus*). The diameter of *A. alternata* colonies, on

PDA was 4.8–6.8 cm. Colonies of *A. alternata* from *A. paniculatus* were light grey, those from *A. cruentus* were usually grey, and those from *A. retroflexus* varied from light grey, through grey, to dark grey.

Jadhav et al. (2011) studied morphology of ten *Alternaria macrospora* isolates. The mycelium was septate, hyaline and branched with 3.0 to 3.40 µm in width. The size of conidia ranged from 20.81- 56.23 x 9.2- 27.10 µm with 1 to 6 transverse septa and 0 to 4 longitudinal septa. The average maximum conidial length (45.37 µm) and breadth (22.3 µm) observed in I_6 and average minimum length (32.49 µm) and breadth (12.60 µm) observed in isolate I_2. Maximum transverse and longitudinal septa were recorded in isolate I_6 (2-7), (0-4), respectively. Least transverse and longitudinal septation was in isolate I_9 (1-4), I_9 (0-2) respectively.

Khodaei and Arzanlou (2013) 97 isolates of *Alternaria alternata* were recovered from infected leaves of sunflower. Colonies on Potato Carrot Agar were reddish brown to olivaceous grey, flat, circular, entire, reaching a diameter of 40–75mm after one week. Mycelium superficial or submerged, consisting of branched, septate, subhyaline, smooth to verruculose hyphae. Conidiophores mostly solitary, macronematous, brown, simple or branched or with one to several geniculation, (23-) 40-52 (-91)×4-5 µm, producing 25 or more ellipsoidal or ovoid conidia in a branched chain. Conidia straight, pale to dark brown to olivaceous green, smooth or punctuate, (15-) 20-25 (-40)×(7-) 10-11 (-15) µm, with 1–6 transverse septa and 0–3 or rarely four longitudinal septa. Secondary conidiophores aseptate or septate, up to 37×3– 5 µm.

Sofi et al. (2013) studied twenty one isolates of *A. mali* were collected from different locations associated with Alternaria leaf blotch of apple. *A. mali* colonies varied in their cultural behaviour ranging from velvety to cottony, mostly appressed, with regular to irregular margins. Colour of colonies ranged between light to dark olivacious. Isolates impregnated media with colour ranging between grey to brown. Growth rate of isolates was between 5.86 to 8.21 mm/day with fast growth in isolate Am-13 and least in Am-5. Average conidial size ranged from 21.36 to 31.74 x 8.34 to 14.48 µm.

Marak et al. (2014) studied morphological variations of *Alternaria solani* isolated from different solanaceous crops. The conidia varied from 20.68-43.10x10.53-17.99 µm in brinjal, 19.86-43.73x7.52-13.05 µm in chilli, 21.5-33.21x8.03-17.85 µm in potato and 30.31-75.47x7.26-27.42 µm in tomato. Variability with respect to cultural characters showed that brinjal, tomato and potato isolates produce cottony mycelia growth in all the tested media. Maximum growth was recorded on PDA for chilli and potato (93.33 mm and 95.00 mm respectively), CDA for brinjal (85.33 mm) and PCA for tomato (87.00 mm).

Hashem et al. (2014) studied growth of *A*. *alternata* on Potato dextrose agar (PDA), Oatmeal agar (OA), Richards agar (RA), Czapek Dox agar (CDA), and Water agar (WA). The radial growth of *A*. *alternata* maximum 8.85 cm on OA followed by 8.67 cm in PDA. The sporulation of *A*. *alternata* was maximum ($3.69\times10^6/cm^2$) on WA followed by RA ($2.44\times10^6/cm^2$). The pure culture of the fungal colony appeared to be grayish white at first and became black later on. The fungus produced abundant conidia having 3-8 transverse septa and 1-2 longitudinal septa.

Singh et al. (2014) studied morphological characteristics of ten isolates. Radial growth in isolate RF-1 was 35.50 mm, while isolate EC-1 recorded maximum i.e 52 mm after 10 days inoculation. In case of IIVR and BHU-1 recorded 88.75 mm radial growth after thirteen days of inoculation. The maximum mean mycelial growth was observed in isolate IIVR (57.83 mm) followed by MF-4 (57.66 mm) and BHU-1 (56.83 mm). Mycelia growth pattern were observed on PDA where BG RF-1, SF-1, MF-4, BHU-1 grew with circular margin with smooth surface colony and AF-2, PN-4, EC-1, BX-2 and IIVR isolates grew with irregular margin and rough surface.

Ginoya and Gohel (2015) studied variability among the isolates of *A. alternata* on seven different media. Oatmeal agar and potato dextrose agar were found as an excellent media to support the growth and spore formation of isolates of *A. alternata*. In case of isolates, Ahmedabad (Aa-7) and Rajkot (Aa-8) mycelial growth was 61.90 mm and 61.71 mm. The sporulation was found abundant in Anand isolate (Aa-1). The average conidial length varied from 16.93 to 59.24 µm and breadth ranges from

6.90 to 14.98 µm with beak length of 3.25 to 44.07 µm. The transverse and longitudinal septa varied from 2 to 10 and 0 to 4, respectively.

Zahra Ibrahim El-Gali (2015) isolated *A. alternata* from carob (*Caratonia siliqua* L.) produces grayish black mycelium with very thin white margin and tints of olive or brown on PDA plate. Colonies are spreading hairy and grey brown to black, possessing a texture similar to cotton, felt or velvet and then become dark green. The fungus produced straight or curved primary conidiophores, short to long, simple or branched, with one or several apical conidiogenous loci. Conidia are obclavate, long ellipsoid, small or moderate in size, septate and medium-brown to dark olive, with 2 to 7 transverse and 0 to 2 longitudinal or simple or branched chains. Secondary conidiophores can be formed apically or laterally with one or a few conidiogenous loci. The length of their conidia was varied from15.1 to 29.1 µm. The longest conidia was in A2 (29.08 µm) followed by A3 (22.9 µm). The conidium was the shortest in A1(15.10 µm). Among all the isolates, the conidia of A4 had 2-9 cells per conidium.

Zhao et al. (2016) isolated *A. alternata* causing leaf blight in watermelon, had dark olivaceous colonies on PDA plates. Conidia were ovoid, ellipsoid or obpyriform with 1-4 transverse and 0-3 longitudinal septa, and measured 21.5-33.7×7.6-11.8 µm. These isolates usually produced conidial chains with numerous secondary and occasionally tertiary chains on PDA plates.

Vrushali Gagrepatil and Vanmare (2016) studied the incidence of fungal diseases of capsicum in relation to seasonal variation, found that *Alternaria solani* causes infection to leaf and stem. In culture, colonies were olive green or brownish black in colour.

Devappa and Thejakumar (2016) studied the cultural characteristics of *A. alternata* on eleven different media. Maximum radial growth was observed on Richard's agar (90.00 mm) which was on par with oat meal agar (89.00 mm). The maximum sporulation was observed in Potato dextrose agar followed by Richards's agar, Oat meal agar and Asthana and Hawker's medium. Colony was light grey to dark grey colour

in all media except in Richard's agar wherein cream/whitish colour of the colony was noticed.

Devi et al. (2016) studied growth characters of ten isolates of *A. helianthi* on six solid media and tested PDA recorded significantly the maximum mycelia growth (7.64 cm) followed by sunflower leaf extract agar (6.95 cm). The Colonies of *A. helianthi* on PDA light brown to olive brown, velvety, slowly growing 15-20 mm (in darkness) or 35 mm (under light).

2.5 Molecular variation

Morris et al. (1999) studied 69 isolates of A. alternata for genetic variation by using 29 primers. Total 137 bands were generated, out of which 103 bands were polymorphic. The size of amplification product ranging between 200 to 1600 bp. The 69 isolates were clustered into two major phenetic groups (Gp1 with 55 isolates and Gp2 with 14). The genetic similarity within groups (73% for Gp1, 80% for Gp2) was much higher than the 50% similarity between the two phenetic groups.

Pryor and Michailides (2002) in RAPD analysis among thirty isolates of *Alternaria*, a total 136 RAPD fragments were scored with little variation among isolates of *alternata* and *tenuissima* species group. For PCR-RFLP analysis, primer directed amplication of a 2500-2700 bp fragment from all isolates tested. All product were reproducible and a total 64 DNA fragment were scored, cluster analysis of RAPD and PCR-RFLP reveled three cluster. First cluster containing infectoria species group. Second cluster containing arborescens species-group and third cluster containing *alternata* and *tenuissima* species group. There was 88% similarity in RAPD and PCR-RFLP fragment pattern was recorded among isolates of infectoria species group. Sequence analysis of ITS region reveled 100% sequence identify among the eight pistachio isolates.

Gherbawy (2005) studied nine different species of *Alternaria* represent by 27 isolates collected from several Egyptian crops and analyzed by RAPD-PCR. Product amplified between the range of 400 bp to 2000 bp. Genetic similarity between isolates of *A. alternata* species-group ranged from 14.8 to 100%, whereas the range was from 14.81 to 96.30%

between the other species used in this study. The dendrogram showed that most isolates cluster according to the source of isolation with few exception. RAPD I cluster includes isolates 18, 17, 15, 11 and 16 (isolated from tomato except isolate 11 isolated from maize). RAPD II cluster include isolates 14, 13, 12 and 4 (isolated from bean except isolate 4 isolated from wheat). RAPD III cluster include isolate 10, 7, 3, 2 and 1(citrus isolates except isolate 7 from sugarcane). RAPD IV cluster include isolates 9 and 8 isolated from sugarcane 5 and 6 from lentil.

Francisco et al. (2009) studied molecular characterization by RAPD analysis in twenty four isolates of *A. alternata* isolated from mango. A total of 85 bands ranging from 0.2 to 3.0 kb were observed. Out of 85 bands, 71 were polymorphic among the isolates fingerprints, while the remaining 14 bands revealed to be monomorphic. 83.52% polymorphism was observed in these analysis. In Principal Components Analysis four group were observed, formation of groups showed correlation with the host plant. Only exception was the cluster II: LRS 23/4, LRS 25/4, LRS 26/4 and LRS 27/4. obtained from lemon plant. Other three observed groups were the cluster I, the biggest group composed by all isolates from tangor "Murcott" and other additional isolates (LRS 04/03, LRS 25/03, LRS 35/03, LRS 36/03, LRS 38/03, LRS 43/03). The cluster IV showed to be constituted by two endophytic isolates from mango (14M and 16M) and one pathogenic isolate from tangerine "Cravo". At last, the cluster III was not clearly formed in RAPD analysis, with samples LRS 37/3, LRS 39/3, 12M and 25M spread in the biplot.

Jadhav et al. (2011) studied genetic variation among 10 isolates of *A. macrospora* by using 10 RAPD primer. A total 101 amplicons were synthesized by amplification. Out of which, 80 were polymorphic with an average of 79.20% polymorphism. Each primer produced on an average 10.1 amplicons. The size of the amplified product ranged from 250-2700 bp. The Jaccard similarity coefficient values among ten isolates of *A. macrospora* ranged from 0.25 to 0.70. The UPGMA based dendrogram separated these isolates into two major clusters. Cluster I comprised isolates I_1, I_2 and I_7. Cluster II comprised of seven isolates consisted of

isolate I_3, I_4 I_6, I_8, I_{10}, I_9 and I_5. Cluster I comprised isolates I_1 and I_2 collected from Ahmadnagar district of western Maharashtra and isolate I_7 collected from Aurangabad district of Marathwada region. Cluster II consisted of isolate I_3, I_4, I_6 I_{10}, I_8, I_9 and I_5 which were collected from Pune, Dhule, Akola, Amravati, Bhalgaon, Nagpur and Agriculture Research Station. Pune of western Maharashtra and Dhule of Khandesh region, respectively showed maximum similarity. In cluster II about 70 per cent similarity coefficient was observed between isolate I_{10} and I_8 representing Vidarbh region.

Nasim (2012) used Random amplified polymorphic DNA technique (RAPD) was used to determine the finger print of ten isolates of *Alternaria alternata* collected from First Fungal Culture Bank of Pakistan (FCBP). Among ten, Five isolates were able to produce clear DNA bands, were Aa1, Aa2, Aa3, Aa4, and Aa5. A total of 22 bands ranging in size from 250-1200 bp were generated by the primer GL-A-01. Nineteen of these 22 bands (86.36%) were monomorphic. Only three bands with sizes of 250, 600 and 1200 bp produced by primer GL-A-01, were polymorphic. Cluster analysis of similarity between band pairs showed that the fungal isolates were clustered in two distinctive groups. Dendrogram obtained by primer GL-A-01 formed one cluster in which Aa1 was grouped with Aa2 were 86.51% similar. Aa1 and Aa2 were 100% similar to Aa3. Aa3 and Aa4 was formed one subcluster and are 58% similar. Aa4 was nearer to Aa5 genetically. Aa5 show 45.55% similarity towards Aa1, Aa2, Aa3 and Aa4.

Sofi et al. (2013) studied genetic variation of twenty one isolates by using 4 RAPD primers. Total 42 band produced by primer, out of which 41 bands were polymorphic. Thus, 96.61% polymorphism observed in these analysis. The dendrogram analysis showed 57.5% Dice similarity coefficient all the isolates formed a single clade, thus showing 42.5% dissimilarity among the isolates. At 68%, all the isolates were categorized into 5 clusters (I to V). Cluster I was further subdivided into Ia and Ib groups accommodating 9 and 2 isolates, respectively.

Tiwari (2013) studied genetic variation of isolates of *A. alternata* isolated from karanj through RAPD primers. Total number of band

generated by 2 amplifying primer was 23, out of which 6 bands were polymorphic produced by GCC 181 and GCC 180. The average polymorphism generated by these bands was 48.75%. The size of amplification products ranged between 264 to 21226 bp. Dendrogram showed that the total isolates categorized into three group. First group consist of twelve isolates second group consist of four isolates and third group consist of one isolate. A1 2 and A1 4(Group first) and A1 9 and A1 11(Group second) have similarity value more than 0.9, showed that these isolates are genetically near to each other.

Pratibha Sharma (2013) studied genetic variation among 32 isolates of *A*. *brassicae* analyzed with Random Amplified Polymorphic DNA. The products amplified between the range 150-900 bp. in which the mean similarity coefficient was found to be 0.73 and 0.84, respectively. Further internal transcribed spacer analysis showed all isolates were 90–100 % similar to each other.

Ginoya and Gohel (2016) studied genetic diversity of eight isolates of *Alternaria alternata* collected from Gujarat. Only 10 of the 65 RAPD primers were selected based on repeatability. The 10 RAPD primers produced 211 bands, with fragments size ranging from 119.93 to 3236.45 bp. Out of these, 98 were polymorphic giving 98.98 per cent polymorphism. The average polymorphic bands per primer were 9.8 and percent polymorphism ranged from 85.71 in OPF-1 to 100 in rest of the primers. Dendrogram based on Jaccard's similarity coefficient formed two clusters namely 'A' and 'B'. The cluster 'A' included Aa-1 and Aa-2 isolates, while the cluster 'B' included Aa-3, Aa-4, Aa-5, Aa-6, Aa-7 and Aa-8 isolates. Similarity coefficient value ranged between 0.31 to 0.061. maximum similarity coefficient value 0.31 was observed between Aa-2 (Kheda) and Aa-1(Aanand). The least similarity index value 0.061 was observed between Aa-8 (Rajkot) and Aa-1(Aanand).

Zhao et al. (2016) studied PCR amplification of the rDNA- ITS region generated 570 bp fragment for all the *Alternaria* isolates. Fourty nine isolates produced 546 bp fragment known *A. tenuissima* isolates retrived form genebank database. The remaining fifteen isolates obtained a 440 bp fragment of *A*. *alternaria* isolates.

CHAPTER III

MATERIAL AND METHODS

During the course of present studies the details of materials used and methods implemented are presented in this chapter. All *In vitro* studies on fruit rot pathogen and molecular analysis work were conducted in the laboratory of Department of Plant Pathology, Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola.

3.1 Materials

3.1.1 Glasswares

The glasswares *viz.*, petri dishes, conical flasks (viz., 100 ml, 250 ml, 500 ml, 1000 ml and 2000 ml), test tubes, pipettes, beakers, slides, cover slips, glass rods, measuring cylinders (10 ml, 100 ml, 250 ml and 1000 ml), reagent bottles and micropipettes were used.

3.1.2 Equipments

The laboratory equipments *viz.,* Autoclave, Hot Air Oven, Laminar Air Flow, BOD incubator (Remi), Research microscope, Stereoscopic microscope, Digital weighing balance (Wensar HBT 516), Centrifuge machine (Eppendorf 5810R), PCR machine (Eppendorf GR), Gel electrophoresis (Genexy, Scie-Plas), gel doc machine, vortex (Spinix), ultra low deep freezer -80^{0}C, deep freezer -20^{0}C, Freezer, Digital camera, Bioage water purifier, Double distillation unit, Ice maker etc. were used.

3.1.3 Isolates

The isolates of *Alternaria alternata* from Kolhapur, Satara, Sangli, Akola, Nagpur and Amravati were collected.

3.1.4 Miscellaneous material

Inoculation needle, single and double distilled water, spirit lamp, spirit, scalpel, forcep, cork borer, haemocytometer, eppendorf tube, PCR tubes and tips, blotter paper, non-absorbent cotton, plastic pots, dissection needle, tray, rubber bands, mortar pestles, scissors etc. were used during the studies.

3.1.5 Culture Media

Potato Dextrose Agar (PDA) and Potato Dextrose Broth (PDB) were used during laboratory studies of fungus.

3.2 Methods

3.2.1 Sterilization of glassware, media, water, blotter paper and other material

The petri plates, test tubes, reagent bottles and conical flasks of different capacities i.e 1000ml, 500ml, 250ml etc. were sterilized in Hot air oven at 180^0C for one hour, whereas, the media, distilled water and blotter paper were sterilized in autoclave at 15 psi for 15 minute. The material *viz.,* needles, inoculating needle, forcep and scalpel were sterilized on flame by direct heating.

3.2.2 Precautions to eliminate contamination

All the isolation and inoculation work of microbial culture was carried out under aseptic condition. The laminar air flow was sterilized by glowing ultraviolet light for half an hour prior to commencement of work. The working surface and side glasses of laminar air flow were sterilized with denatured spirit.

3.2.3 Collection of disease samples from different chilli growing area of Maharashtra

Table 1. Diseased samples collected from different locations

Sr. No.	Locations	Sample detected
1	Kolhapur	Isolate (Aa1)
2	Satara	Isolate (Aa2)
3	Sangli	Isolate (Aa3)
4	Akola	Isolate (Aa4)
5	Nagpur	Isolate (Aa5)
6	Amravati	Isolate (Aa6)

3.2.4 Procedure for preparation of PDA media

Table 2. The composition of Potato Dextrose Agar medium

Sr. No.	Ingredient	Quantity
1	Peeled and sliced potato	200 gm
2	Dextrose ($C_6H_{12}O_6$)	20 gm
3	Agar	20 gm
3	Distilled Water	1000 ml

1. The potatoes were peeled, cleaned under running water and sliced into pieces.
2. Then 200 gm peeled potato were taken and boiled in 500 ml sterilized distilled water in saucer pan for 30 min until they get soft.
3. In another 500 ml of water, 20 gm agar and 20 gm dextrose was added and dissolved. The potatoes were stained through muslin cloth and the extract was collected.
4. Both parts were mixed and made final volume 1 litre. Then the media thus prepared was dispensed in conical flask to half of its capacity.
5. The flask were plugged with non absorbent cotton and covered by paper. The potato dextrose broth was prepared without adding agar agar. The flask were then autoclaved at 15 psi for 15 minutes.

3.2.5 Procedure for isolation of *A. alternata*

Chilli fruit showing the typical symptoms of fruit rot were collected from different places of Maharashtra state. The infected lesions were cut into small pieces by means of a sterile scalpel and surface sterilized in 0.1 per cent mercuric chloride solution for 30 sec. and washed repeatedly by using sterile distilled water. Then the bits were placed onto sterilized petri plates containing solidified PDA medium under aseptic conditions in the culture room. The plates were incubated at room temperature (28 ± 2^0C) for five days after incubation. The tip of hyphal

growth radiating from the infected tissue was transferred onto PDA petri plate.

3.2.6 Maintainance of *A*. *alternata* isolates

The single spore of each *A*. *alternata* isolate was sub cultured on PDA slants and allowed to grow at 27 ± 2°C for 10 days and the slant were preserved in a refrigerator at 4°C and revived frequently.

3.2.7 Pathogenicity test

Healthy riped chilli fruits collected and were surface disinfected with sodium hypochloride for two minutes and then rinsed with three washes of sterilized distilled water. These healthy fruits were pin pricked with sterilized needle prior to inoculation. Three pricks were given on the ripe fruit at top, middle and lower portion of the fruits. The conidial suspension having 1×10^5 spores/ml applied to the pin pricked fruits with the help of sterilized cotton swabs. The inoculated fruits were placed in moist chamber and incubated at room temperature. Initiation of typical symptoms after 3-5 days on the fruits, the diseased portion cut into small pieces along with healthy part and resorted for reisolation and compared with the original isolates. The diseased development was recorded by measuring lesion length of the diseased portion, days to initiate the symptoms and complete rotting of fruits was recorded after inoculation.

3.2.8 Growth pattern study of *Alternaria alternata* on different media

All the 6 isolates of *Alternaria alternata* were tested for their growth characteristics i.e. radial growth, colony colour, colony margin, shape and size of conidia etc. on five different media *viz.*

1) Potato Dextrose Agar Media

1)	Peeled potato	200 g
2)	Dextrose	20 g
3)	Agar-agar	20 g
4)	Distilled water	1000 ml

2) Oat Meal Agar Media

1)	Oat meal	60 g
2)	Agar-agar	20 g
3)	Distilled water	1000 ml

3) Czapek's Dox Agar Media

1)	Sucrose	30 g
2)	Sodium nitrate	2 g
3)	Dipotassium phosphate	1 g
4)	Magnesium sulphate	0.50 g
5)	Potassium chloride	0.50 g
6)	Ferrous sulphate	0.01 g
7)	Agar- agar	20 g
8)	Distilled water	1000 ml

4) V-8 Juice Agar Media

1)	V-8 juice (100 ml)	8.30 g
2)	L-Asparagine	10 g
3)	Yeast extract	2 g
4)	Caicium carbonate	2 g
5)	Glucose	2 g
6)	Agar-agar	20 g
7)	Distilled water	1000 ml

5) Corn Meal Agar Media

1)	Corn meal	50 g
2)	Agar-agar	20 g
3)	Distilled water	1000 ml

Note: Degree and categories of sporulation

Rate of sporulation	No. of spores / microscopic field	Sporulation category
Abundant	> 30	4
Good	21 – 30	3
Moderate	10 – 20	2
Scanty	< 10	1
Nil	0	0

3.2.9 RAPD analysis of *Alternaria alternata* isolates

The total 6 isolates of *Alternaria alternata* were used for PCR-RAPD analysis. The following reagent/chemicals and their composition were used.

Chemicals

Chemicals used for genomic DNA extraction, Polymerase Chain Reaction and electrophoresis are listed as below.

A) Stock Solutions

A) Tris HCl (100mM pH 8.0)

Tris HCl	1.576 g
Distilled water	100 ml

B) Tris HCL (1000mM pH 8.0)

Tris HCl	15.76 g
Distilled water	100 ml

C) Nacl (5000 Mm pH8.0)

Nacl	29.22 g
Distilled water	100 ml

D) EDTA (100 Mm)

EDTA	2.995 g
Distilled water	100 ml

B) Extraction Buffer

A) CTAB 2%

CTAB	3 g

B) Nacl 1.4 m

Nacl	12.2724 g

C) EDTA 20 Mm

EDTA (100 Mm)	30 ml

D) Tris HCl 100 mM

Tris HCl 1000 mM	45 ml

The Tris HCl and EDTA was taken in measuring cylinder from their respective stock solutions. In this solution Nacl and CTAB was added and then the volume make up to 100 ml by addition of distilled water.

C) $T_{10}E_1$ buffer

Tris HCl (100Mm)	1 ml
EDTA (100Mm)	0.1 ml
Distilled water	8.9 ml

Tris HCl and EDTA was taken in measuring cylinder and then volume make up to 10 mlwas done by addition of distilled water.

D) 70 % Ethanol

Ethanol	70 ml
Distilled water	30 ml

E) RNAse (Diluted RNase)

RNase	43 µ
Distilled water	172 µ

10.51 µ from this stock solution used per 200 µ of DNA sample.

F) 10X TBE (Tris-Borate EDTA)

Tris base	108 g/lit
Boric acid	55 g/lit

0.5 M EDTA (pH-8.0)	40 ml/lit
Distilled water	1 lit

G) Liquid nitrogen

H) Dichloroform : Isoamylalcohol (24:1)

I) Isopropanol

J) Phenol : Chloroform, Chloroform

L) Absolute ethanol

M) $Mgcl_2$ (25mM)

N) Loading dye (6X)

O) Ethidium bromide (0.5 mg/ml)

P) dNTPs (2mM)

Q) Taq DNA polymerase (5 units)

R) Primers (RAPD)

S) Nuclear free water

T) Genomic DNA

3.2.10 Selection of isolates for molecular study

The total six isolates of *Alternaria alternata* were used for PCR-RAPD analysis as listed in Table 1

3.2.11 Procedure for Genomic DNA Extraction

The DNA was extracted by the method of Coddington and Gould, (1992). The procedure of DNA extraction was carried out in two ml eppendorf tube.

1) The pure culture of fungus grown on potato broth 200 ml in 500 ml conical flask for seven days at a temperature of 27± 2°C in BOD incubator.
2) The mycelial mat was harvested after seven days.

3) It was wash thoroughly and repeatedly and then dried using blotter paper and crushed to powdered form in pre-chilled pestle and mortar with liquid nitrogen.

4) The powdered mass was immediately homogenized by adding pre-warmed (65^0 C) 1 ml of CTAB extraction buffer (100 mM Tris HCl pH 8.0, 20 mM EDTA 1.4 M NaCl, 0.4 % β-mercaptoethanol and 2 % w/v CTAB) per tube and the content was mixed gently by inversion.

5) The mixture was incubated at 65^0 C for one hour in hot water bath with intermittent shaking by gently inverting the tube after 10 minutes.

6) The tubes containing homogenate were centrifuged at 8000 rpm for 15 minutes.

7) The supernatant was transferred into another 2 ml eppendorf tubes without disturbing the pellet of cell debris.

8) Then equal volume (1 ml) of chloroform isoamylalcohol (24:1) was added and mixed gently but thoroughly to emulsify both the components for five minute.

9) Centrifugation was carried out at 12000 rpm for 15 min.

10) The upper aqueous phase was transferred into another 2 ml eppendorf tube.

11) Equal volume of ice-cold isopropanol was added and mixed by inversions. CTAB-DNA complexes formed at the bottom of the eppendorf tubes.

12) After mixing with isopropanol, the sample were kept at 4^0 C for 10 minutes and then centrifuged at 10000 rpm for 10 minutes.

13) After centrifugation a pellet was formed at the bottom of the eppendorf tube.

14) The supernatant was removed and the pellet was washed with 70 % ethanol twice and centrifuged at 8000 rpm for 5 minutes.

15) The pellet was air-dried for 30-60 minutes and then dissolved in 0.5 ml of TE buffer.

16) The pellet were allowed to dissolved completely overnight at 4^0C without agitation.

3.2.12 DNA quantification

1) The DNA obtained after extraction was confirmed by running it on 0.8% agarose gel containing ethidium bromide 0.5 mg/ml in a horizontal gel electrophoresis system.
2) 2 µl of genomic DNA of each isolate + 1.6 µl sterilized water loaded in each well.
3) After completion of 5 cm run, the gel was observed under UV light and the DNA yield and quality was confirmed.

3.2.13 Internal Transcribed Spacer (ITS) amplification

Genetic variability of *Alternaria alternata* was evaluated by using ITS primers (Table 3).

Table 3: List of ITS primers used with their sequences

Oligo Name	Primer Sequence (5'-3')	GC %
ITS 1	TCCGTAGGTGAACCTGCGG	63
ITS 4	TCCTCCGCTTATTGATATGC	50

3.2.10.1 Procedure for PCR reaction

Sterile PCR tubes were numbered and placed on PCR tube stand. At first 2 µl of DNA was added to each PCR tube followed by master mix given in Table 4 and 5. The samples were mixed by brief centrifugation to bring down the content of tube. PCR were run on the programmable thermal cycler given in Table 6. PCR products were separated by electrophoresis in 2 per cent agarose gels run in 1x TBE, stained with ethidium bromide and visualized with a UV transilluminator.

Table 4: PCR reaction mix for 1x of 12.5 µl reaction

Sr. No.	Master Mix	1x
1	10x *Taq* buffer	1.25 µl
2	$MgCl_2$ (25 mM)	1.25 µl
3	dNTPs (10 mM)	0.3 µl
4	*Taq* polymerase (5 U/µl)	0.3 µl
5	Sterile distilled water	5.4 µl
	Total Volume	**8.5 µl**

Table 5: Constituents of PCR reaction for ITS

Sr. No.	PCR Reaction	Quantity
1	Master Mix vol.	8.5 µl
2	Primer (Forward)	1.0 µl
3	Primer (Reverse)	1.0 µl
4	Template DNA (37.5 ng)	2.0 µl
	Total Reaction Volume	12.5 µl

Table 6: Steps used for PCR-ITS reaction

Name of step	Temperature	Time
Initial Denaturation	94°C	5 min.
30 cycles - Denaturation	94°C	1 min.
30 cycles - Annealing	55°C	1 min.
30 cycles - Extension	72°C	30 sec.
Final Extension	72°C	10 min.

3.2.14 Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) polymerase chain reaction (RAPD-PCR) procedure was performed as previously described method by Williams et al. (1990) with some modification in a reaction mixture given in Table 7 and 8. Amplification products were separated on 1.2% agarose gel in 1x TBE buffer at 70 V for about 2 hour.

Table 7: PCR reaction mix for 1x of 20 µl reaction

Sr. No.	Master Mix	1x
1	10x *Taq* buffer	2.0 µl
2	$MgCl_2$ (25 mM)	2.0 µl
3	dNTPs (10 mM)	0.5 µl
4	*Taq* polymerase (5 U/µl)	0.2 µl
5	Sterile distilled water	12.3 µl
	Total Volume	**17 µl**

Table 8: Constituents of PCR reaction for RAPD

Sr. No.	PCR Reaction	Quantity
1	Master Mix vol.	17 µl
2	Primer	2.0 µl
3	Template DNA (37.5 ng)	1.0 µl
	Total volume	20 µl

The PCR tubes containing reaction mixture were placed in the thermal cycler for 40 cycles with the following profiles-

Table 9: Steps used for PCR-RAPD reaction

Name of step	Temperature	Time
Initial Denaturation	94°C	5 min.
30 cycles - Denaturation	94°C	1 min.
30 cycles - Annealing	34°C	1 min.
30 cycles - Extension	72°C	30 sec.
Final Extension	72°C	10 min.

Table10: List of primers used for RAPD analysis

Sr. No.	List of primers	Sequence	Annealing temperature(°C)
1	OPA-1	CAGGCCCTTA	34
2	OPA-2	TGCCGAGCTG	34
3	OPA-3	AGTCAGCCAC	34
4	OPA-4	AATCGGGCTG	34
5	OPA5	AGGGGTCTTG	34
6	OPA-6	GGTCCCTGAC	34
7	OPA-9	GGGTAACGCC	34
8	OPA-10	GTGATCGCAG	34
9	OPA-12	TCGGCGATAG	34
10	OPA-13	CAGCACCCAC	34
11	OPA-14	TCTGTGCTGG	34
12	OPA-15	TTCCGAACCC	34
13	OPA-18	AGGTGACCGT	34
14	OPA-19	CAAACGTCGG	34
15	OPB-11	GTAGACCCGT	34

3.2.15 Electrophoresis of RAPD-PCR

The RADP analysis was carried out in horizontal gel electrophoresis. The PCR products were separated electrophoretically in 1.2% agarose gel using 1X TAE buffer. The gel was stained with ethidium bromide.

The cleaned and dried electrophoresis assembly was used for RAPD. The gel tray was wiped and cleaned with ethanol. The agarose gel solution was prepared by mixing agarose gel in 1X TAE buffer, this mixture was heated in microwave oven for 3 min. the ethidium bromide was added to the gel solution as staining agent. The gel solution was then poured in the gel casting tray and combs was placed in the gel and allowed to set. 1X TAE buffer was used as the tank buffer. After the casting of the gel the combs were removed and the gel was placed in electrophoresis assembly with 1X TAE buffer. The gel was pre-runned for 15 min. the care was taken while handling the gel as the ethidium bromide added is highly mutagenic. After the pre-run, the RAPD-PCR product was mixed with 5 µl 6X dye and the mixture was loaded in the wells, along with the 10 µl DNA ladder in the first well. The gel was run for 2 hrs. at 70V. after the run, the gel was removed carefully from the unit and observed in Transilluminator under UV light and photographed.

3.2.13 Data analysis

The gel images were captured and visualized in gel documentation system. The data was scored as the presence (1) or absence (0) of individual band for each isolates in RAPD-PCR analysis of isolates of *Alternaria alternata*.

The data was used to generate similarity coefficient using simple matching coefficient based on RAPD bands scoring. The Dice coefficient between each pair of accessions were then used to construct a dendrogram using he Unweigheted Pair Group Method with Arithmetic Average (UPGMA).

CHAPTER IV

RESULTS AND DISCUSSION

Fruit rot of chilli caused by *Alternaria alternata* is one of the major constraint in total production of chilli. Disease causing 5-85 per cent damage in field as well as in storage (Singh, 1987). Chemical control of fruit rot is not feasible and economical because seed-borne nature of the pathogen. The most practical and cost-effective method for management of fruit rot of chilli is the use of resistant cultivars. But there is lack of knowledge of resistance genes and therefore new virulent or aggressive strains of the pathogen reduced the efficiency of resistant cultivars. Hence investigations on molecular characterization of *A. alternata* causing fruit rot of chilli through RAPD marker was taken.

The isolates of *A. alternata* were collected from different district of Maharashtra. The results and its interpretation of the present studies are given in this chapter.

4.1 Collection, isolation, purification and identification of pathogen

Diseased chilli plant parts showed typical fruit rot symptoms such as small, circular necrotic spot with irregular margin and it remained brown to black in colour surrounded by yellow halo were collected from major chilli growing area (Plate1).

Table11: Details of the diseased samples collected from various locations of Maharashtra

Sr. No.	District	Location/ Village	Infected plant part	Fungi obtained	Isolates designated
1	Kolhapur	Panhala, Tal-Panhala	Fruit	*Alternaria alternata*	Aa1
2	Satara	Vaduj, Tal-Khatav	Fruit	*Alternaria alternata*	Aa2
3	Sangli	Malgaon, Tal-Miraj	Fruit	*Alternaria alternata*	Aa3
4	Akola	Chilli Research Unit, PDKV, Akola	Fruit	*Alternaria alternata*	Aa4
5	Nagpur	Nirwa, Tal-Umred	Fruit	*Alternaria alternata*	Aa5
6	Amravati	Partwada, Tal-Achalpur	Fruit	*Alternaria alternata*	Aa6

Plate 1: Diseased sample collected from different locations

Alternaria alternata was the most commonly isolated fungal pathogen from infected chilli plant parts collected from various locations of Maharashtra. The tissue isolation technique was followed to isolate the pathogen from infected plant parts showing fruit rot symptoms.

The pure culture was obtained and purified by using hyphal tip method and identified as *Alternaria alternata* on the basis of morphological characters reported by Ginoya and Gohel (2015). Total six isolates of *A. alternata* were obtained and abbreviated as Aa1 to Aa6 Table 11.

4.2 Morphological characters

The morphological characters of the pathogenic isolates with respect to radial mycelia growth, colony colour, colony margin, and conidial characters were studied on PDA media.

4.2.1 Radial growth rate (mm) and shape of conidia of *Alternaria alternata* on different media

For the morphological characterization such as radial growth (mm) and shape of conidia total five culture media *viz*., PDA, OMA, CDA, V-8 and CMA were used. The results predicted in Table 12 and Plate 3 revealed that, the highest growth (75.65 mm) was recorded on Potato Dextrose Agar media. The second best medium was Oat Meal Agar media (59.4 mm) followed by Corn Meal Agar media (46.6 mm) while minimum radial growth i.e 31.66 mm was recorded in V-8 agar media.

In case of different isolates, maximum radial growth (53.50 mm) was recorded in Sangli isolate (Aa3), followed by Satara isolate Aa2 (52.5 mm). However minimum was observed in Amravati Aa6 i.e 49.00 mm.

These results are in confirmity with the finding of Hashem et al.(2014), who found that maximum growth of *A*. *alternata* on PDA followed by OA, RA, CDA, WA. Ginoya and Gohel (2015), Devappa and Thejakumar (2016) and Devi et al. (2016) also recorded the same observation which confirms the present studies.

Table 12: Radial growth rate (mm) and shape of conidia of *A. alternata* on different media

Radial growth rate (mm) after 7th days of inoculation							Shape of conidia after 7th days of inoculation				
Isolate	PDA	OMA	CDA	V-8	CMA	Mean	PDA	OMA	CDA	V-8	CMA
Aa1	75.4	57.4	43.5	35.7	46.2	51.64	Muriform, broadly rounded base	Muriform, broadly rounded base	Muriform broadly rounded base	Muriform broadly rounded base	Muriform
Aa2	77.3	59.3	40.9	37.6	47.4	52.5	Obclavate blunt at the tip	Oval	Obclavate to oval	Obclavate blunt at the tip	Obclavate
Aa3	76.1	61.3	42.6	36.5	51.1	53.5	Obclavate	Obclavate	Obclavate to oval	Oval	Oval
Aa4	75.3	59.8	43.3	26.5	47.3	50.44	Muriform, rounded base	Muriform	Muriform	Muriform	Muriform
Aa5	75.5	61.7	45.7	29.1	46.4	51.68	Obclavate blunt at the tip	Obclavate to oval shaped	Obclavate	Obclavate blunt at the tip	Obclavate
Aa6	74.3	57.3	47.3	24.8	41.3	49.0	Clavate to oval	Clavate	Oval shape	Oval	Clavate
Mean	75.65	59.4	43.8	31.6	46.6						

Plate 2: Growth of *A. alternata* on PDA, mycelial growth and conidia

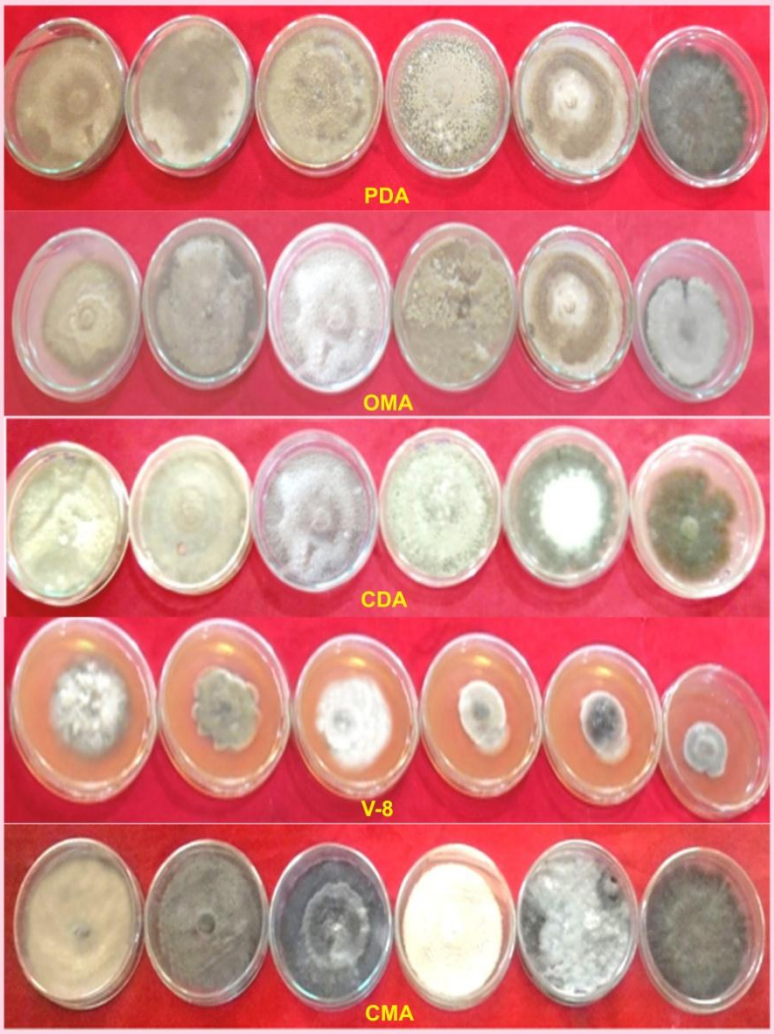

Plate 3: Growth of *A. alternata* on different media

The conidial shape of *A. alternata* were found light to dark brown in colour with muriform, broadly rounded base formed in Aa1 and Aa4 isolates on PDA, OMA, CDA, V-8 and Aa4 on all media. In some isolates Aa2, Aa3 and Aa5 conidia are obclavate to oval in shape with blunt at tip isolates on all media Table 12.

4.2.2 Size and septation of conidia of *A. alternata* on different media

The results presented in Table 13 revealed distinct variation among isolates and media. Among the five different media tested, maximum conidial length (43.30 µm) was noticed in CMA, followed by PDA (42.10 µm) in Aa1 isolate. While, the minimum conidial length (27.7 µm) was noticed in CDA in Aa1 isolate collected from Kolhapur.

Maximum conidial breadth (12.4 µm) was noticed in PDA and OMA in isolate Aa4 followed by Aa2 i.e 12.3 µm in PDA. While, the minimum conidial breadth (8.3 µm) was noticed in CMA in Aa2 isolate followed by 8.80 µm in V-8 and OMA in Aa2 and Aa3, respectively.

In case of conidial septation as presented in Table 13 there was difference among the isolates with respect to transverse and longitudinal conidial septation on different media. The maximum number of transverse septa were observed in Aa1 isolate on OMA media (7-9) and minimum in Aa6 isolate (3-4) on PDA. The maximum (0-3) number of longitudinal septa were observed in Aa2 isolate on PDA, whereas no longitudinal septation was observed in isolate Aa3 on CDA and Aa4 on OMA and CMA media.

Overall average number of septation among the isolates varied from 3-9 transverse and 0-3 longitudinal septa.

These results are in confirmity with findings of Marak et al. (2014) Ginoya and Gohel (2015) who reported differences among the isolates of *A. alternata.*in terms of length, breadth and number of septa.

Table 13: Size of conidia and septation of conidia of *A. alternata* on different media

Isolate	Size of conidia (µm)												Septation of conidia									
	PDA		OMA		CDA		V-8		CMA		Mean		PDA		OMA		CDA		V-8		CMA	
	*L	*B	L	B	L	B	L	B	L	B	L	B	*T	*L	T	L	T	L	T	L	T	L
Aa1	42.1	11.3	41.1	8.9	27.7	10.2	37.8	9.6	43.3	11.4	38.4	10.3	7-8	0-2	7-9	0-1	3-5	0-1	4-5	0	6-7	0-2
Aa2	35.3	12.3	36.6	9.4	34.6	9.5	36.5	8.8	34.5	8.3	35.2	9.7	3-5	0-3	4-7	0-1	4-7	0-2	3-5	0-1	5-6	0-1
Aa3	39.7	10.2	29.8	8.8	29.1	8.9	39.8	9.8	34.9	10.5	33.3	9.6	4-6	0-1	3-5	0-2	3-8	0	3-6	0-2	4-6	0-2
Aa4	41.7	12.4	38.7	12.4	40.6	10.3	41.1	11.2	41.1	12.2	40.6	11.7	3-5	0-2	4-6	0	3-6	0-1	4-5	0-2	3-5	0
Aa5	37.8	10.7	39.7	10.4	32.2	9.9	38.7	10.5	39.1	12.2	38.8	10.9	4-5	0-1	4-5	0-1	4-5	0-1	3-6	0-2	5-7	0-1
Aa6	36.9	9.7	37.5	10.4	36.6	9.5	37.2	10.2	39.9	11.2	37.7	10.2	3-4	0-1	3-6	0-1	4-6	0-1	3-5	0-1	4-7	0-1
Mean	38.9	11.1	37.2	10.5	33.4	9.7	38.5	10.0	38.8	10.9												

*L-Length, B-Breadth, T- Transverse, L-Longitudinal

4.2.3 Colony colour and colony margin of *A. alternata* on different media

Table 14: Colony colour and colony margin of *A. alternata* on different media

Isolate	PDA	OMA	CDA	V-8	CMA
Aa1	Greenish brown with regular margin	Brown with irregular margin	Brownish white with regular margin	Grayish white with irregular margin	Cream white with regular margin
Aa2	Light grey with regular margin	Gray with irregular margin	Greenish brown with regular margin	Brownish gray with irregular margin	Grayish black with regular margin
Aa3	Greenish white with regular margin	Grayish White with regular margin	Grayish white with regular margin	Creamy white with irregular margin	Blackish with regular margin
Aa4	Greenish brown with regular margin	Greenish with regular margin	Creamy white with regular margin	Creamy white with irregular margin	Greenish with regular margin
Aa5	Light brown with white center, white and regular margin	Light brown with white center, white and regular margin	Brownish black with white center and irregular margin	Blackish with irregular margin	Grayish white with irregular margin
Aa6	Grayish black with irregular margin	Gray with irregular margin	Brownish black with irregular margin	Gray with irregular margin	Grayish black with irregular margin

The results are given in Table 14, revealed that there was a considerable variation among colony colour and colony margin on five different media. Among the different isolates, greenish to greenish brown colony colour with regular margin was observed in Aa1, Aa4, Aa2 isolates on PDA, OMA, CDA and CMA media (Plate 3). Whereas, some isolates like Aa2 on PDA, OMA and CMA showed light gray to grayish black colony colour with regular to irregular margin. Whereas, Aa6 showed same colony

colour on PDA, OMA, V-8 and CMA media while Aa1 isolate produce grayish white pigmentation on V-8 media with irregular margin.

Aa5 isolate showed light brown with white center pigmentation having regular to irregular margin on PDA, OMA and CDA. While blackish with regular to irregular margin was observed in Aa5 and Aa3 on V-8 and CMA media. In case of Aa1 isolate, cream white with regular margin was observed in CMA, while Aa4 showed creamy white with regular to irregular margin on CDA and V-8 media.

These results are in confirmity with the findings of Kumar et al. (2008), Sofi et al. (2013), Zahra Ibrahim El-Gali (2015).

4.2.4 Septation of mycelium of *A. Alternata*

All the isolates of *A*. *alternata* produce septate mycelium on five different media (Plate 2).

4.2.5 Sporulation of *A. alternata* on different media

Table 15: Sporulation of *A. alternata* on different media

Isolate	PDA	OMA	CDA	V-8	CMA	Mean
Aa1	3	2	2	1	2	2
Aa2	3	3	2	2	2	2.4
Aa3	3	2	2	0	3	2
Aa4	3	3	2	2	2	2.4
Aa5	3	2	0	1	2	1.6
Aa6	2	2	1	0	1	1.2
Mean	2.8	2.3	1.5	1.0	2.0	

Note: Degree and categories of sporulation

Rate of Sporulation	No. of spores / microscopic field(45×)	Sporulation category
Abundant	> 30	4
Good	20 – 30	3
Moderate	10 – 20	2
Scanty	< 10	1
Nil	0	0

With regard to sporulation, the result presented in Table 15, revealed that good sporulation was in PDA media (2.8) having more than 25 spores in single microscopic field followed by OMA (2.3) having more than 20 spores in single microscopic field . The next best was CMA (2.0) more than 15 spores in single microscopic field followed by CDA (1.5 more than 10 spores in single microscopic field). The sporulation was recorded scanty in V-8 (1.00), having less than 10 spores in single microscopic field.

In case of isolates good sporulation 2.4 was recorded in Aa2 and Aa4. Moderate sporulation (2) in Aa1 and Aa3 isolates followed by Aa5 (1.6), scanty sporulation 1.2 in isolates Aa6.

Based on above results, Potato dextrose agar (PDA) and Oatmeal agar (OMA) were found as an excellent media to support the growth and spore formation of isolates of *A. alternata*, respectively.

Ginoya and Gohel (2015) reported the Oat meal agar and Potato dextrose agar media was the best media to support the spore formation, which confirm the present study.

4.3 Pathogenicity and symptoms

The ripe chilli fruit were disinfected and inoculated with *A. alternata* conidial suspension having 1×10^5 spores/ml applied to the pin pricked fruits with the help of sterilized cotton swabs. Initiation of typical symptoms appears after 3-5 days of inoculation. Formation of small necrotic lesions brown to black in colour and regular to irregular in shape, became sunken lesions and coalesce in severe condition (Plate 4).

Various workers have been reported the pathogenic nature of *A*. *alternata* in chilli fruit rot disease. The findings of Jadhav et al. (2011), Dalphy et al. (2014), Devappa and Zhao et al. (2016) were confirmed by the results the present investigation.

Pathological variation among the isolates of *A*. *alternata*

In order to ascertain the virulence among the isolates of *A*. *alternata* on detached ripe fruits of chilli, observations were recorded after seven days of inoculation.

The data presented in Table 16 and Plate 4, revealed that *A. alternata* isolate Aa5 was the most virulent isolate causing maximum lesion size of 5.5mm with early initiation (4 days) of disease symptoms and partial rotting of fruit within 10 days followed by Aa6 and Aa1 which exhibited lesion size 4.1mm and 3.4mm. The lesion size recorded in Aa3, Aa2 and Aa4 was 2.5mm, 1.9mm and 1.1mm, respectively.

Thus, *A. alternata* isolate Aa5 proved as the most virulent isolate in which maximum lesion size was observed. These findings were similar to the findings of earlier workers, Jadhav et al. (2011) who recorded lesion size (1.9 to 2.1 mm) by pin prick method.

Table 16: Pathological variation among the isolates of *Alternaria alternata*

Sr No.	Isolates	Lesion size (mm)	Days to initiate disease symptoms	Symptoms
1	Aa1	3.4mm	4	Small blackish, circular to elongated spot
2	Aa2	1.9mm	5	Small blackish, circular to elongated spot
3	Aa3	2.5mm	5	Small blackish, circular to elongated spot
4	Aa4	1.1mm	6	Small blackish, circular to elongated spot
5	Aa5	5.5mm	4	Small blackish, circular to elongated spot
6	Aa6	4.1mm	3	Small blackish, circular to elongated spot

After appearance of typical symptoms on chilli fruits the infected fruits were collected and resorted for reisolation.The fungus reisolated from artificially inoculated host (*A. alternata*) was identical to the original one in cultural and morphologically characters. The pathogenicity of the test fungus on chilli fruits was proved with positive result (Plate 4). Thus it was evident that *A. alternata* was pathogenic to chilli causing fruit rot.

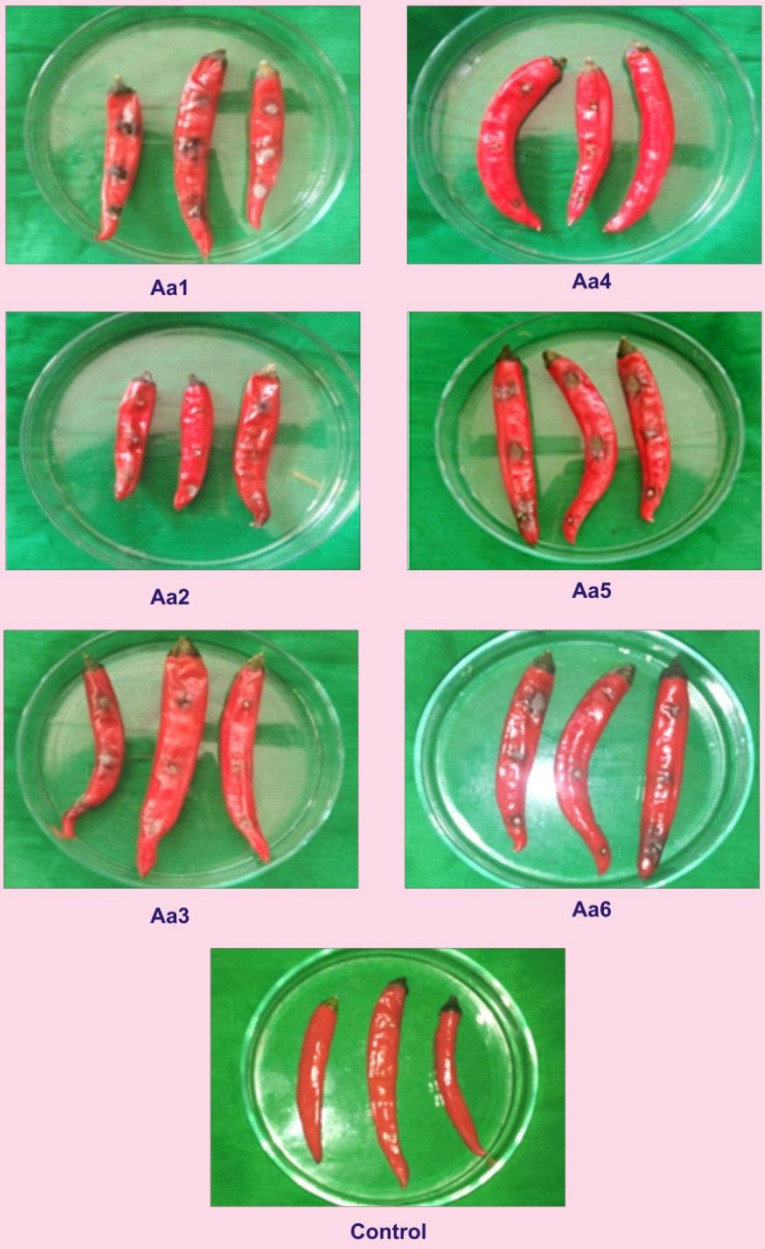

Plate 4: Pathogenicity test of *Alternaria alternata*

4.4 Molecular variation

4.4. 1 *Alterrnaria alternata* selected for molecular study:

The isolates of *Alternaria alternata* were obtained from different agro climatic regions of Maharashtra. The six isolates viz., Kolhapur (Aa1), Satara (Aa2), Sangli (Aa3), Akola (Aa4), Nagpur (Aa5) and Amravati (Aa6), were selected for the analysis. The *Alternaria alternata* specific ITS primers pair ITS-1 (TCCGTAGGTGAACCTGCGG) and ITS-4 (TCCTCCGCTTATTGATATGC) were used for molecular characterization of the isolates. All the isolates of *Alternaria alternata* yielded the 440 bp band with the ITS marker (Plate 6), therefore the results are confirmed with Zhao et al. (2016).

Table 17: Per cent polymorphism observed in RAPD primers

Sr. No.	Primer	Total bands	Polymorphic bands	% Polymorphism
1	OPA-2	8	7	87.5%
2	OPA-3	7	7	100%
3	OPA-6	4	4	100%
4	OPA-10	7	7	100%
5	OPA-12	5	5	100%
6	OPA-13	15	14	93.33%
7	OPA-14	12	11	91.66%
8	OPA-18	7	7	100%
9	OPA-19	11	11	100%
10	OPB-11	7	7	100%
Total		83	80	96.38%

4.4.2 RAPD primers selected for the study

The 15 RAPD primers supplied by “Genaxy” were used to evaluate the molecular variability in six isolates of *Alternaria alternata*. The PCR (Polymerase Chain Reaction) amplified product of each primer were resolved on 1.2% agarose gel electrophoresis and the size of the amplified product was compared with 100+500 bp DNA ladder.

Out of fifteen primers screened for RAPD during present study, 10 primers were polymorphic and primers OPA-1, OPA-4, OPA-5,

OPA-9 and OPA-15 observed no banding pattern for the set of six isolates of *Alternaria alternata.*

RAPD banding pattern

The polymorphic banding pattern was observed in 10 primers. The genetic variability in *Alternaria alternata* was observed in the RAPD banding pattern is given below.

The banding pattern observed in primer OPA-2 is presented in Plate 7. The primer amplified in eight amplicons among six isolates of *Alternaria alternata.* The size of amplicons amplified ranges from 6009 to 207bp. The polymorphism observed in this primer was 87.5 %. The details of the 8 RAPD band types are as follows.

Band type 1 (6009 bp) : This type of band observed in Aa2, Aa4, Aa5 and Aa6 isolates.

Band type 2 (1280 bp) : This type of band observed in Aa2, and Aa4 isolates.

Band type 3 (1197 bp) : This type of band observed in Aa2 isolate.

Band type 4 (490 bp) : This type of band observed in Aa1, Aa2, Aa3, Aa4, Aa5 and Aa6 isolates.

Band type 5 (410 bp) : This type of band observed in Aa6 isolate.

Band type 6 (344 bp) : This type of band observed in Aa2, Aa3, and Aa4 isolates.

Band type 7 (260 bp) : This type of band observed in Aa5 isolate.

Band type 8 (207 bp) : This type of band observed in Aa1, Aa2 and Aa3 isolates.

The banding pattern observed in primer OPA-3 is presented in Plate 8. The primer amplified in seven amplicons among six isolates of *Alternaria alternata.* The size of amplicons amplified ranges from 6005 to 326 bp. The polymorphism observed in this primer was 100 %. The details of the 7 RAPD band types are as follows.

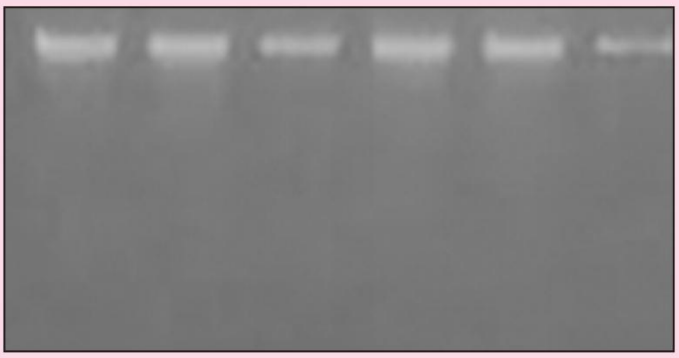

Plate 5: DNA on 0.8% agarose gel

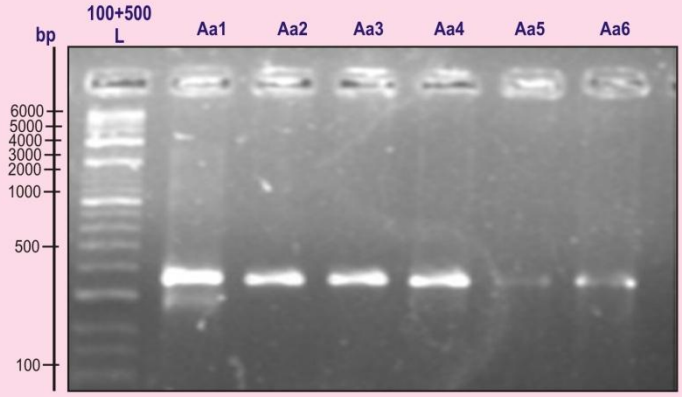

Plate 6 ITS banding pattern of primer

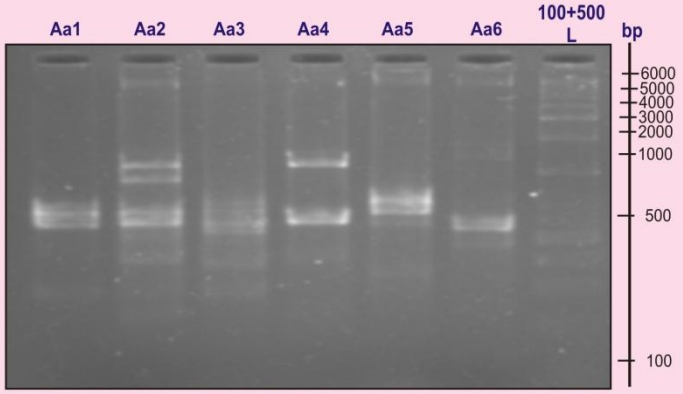

Plate 7: RAPD banding pattern of primer OPA-2

Band type 1 (6005 bp) : This type of band observed in Aa2, Aa3, Aa5 and Aa6 isolates.

Band type 2 (1367 bp): This type of band was observed in Aa2, Aa3 and Aa5 isolates.

Band type 3 (1280 bp): This type of band was observed in Aa2, Aa4 and Aa5 isolates.

Band type 4 (780 bp): This type of band was observed in Aa2, Aa4, Aa5 and Aa6 isolates.

Band type 5 (395 bp): This type of band was observed in Aa2 and Aa4 isolates.

Band type 6 (345 bp): This type of band was observed in Aa3 isolate.

Band type 7 (326 bp): This type of band was observed in Aa3, Aa5 and Aa6 isolates.

The banding pattern observed in primer OPA-6 is presented in Plate 9. The primer amplified in four amplicons among six isolates of *Alternaria alternata.* The size of amplicons amplified ranges from 5895 to 205 bp. The polymorphism observed in this primer was 100 %. The details of the 4 RAPD band types are as follows.

Band type 1 (5895 bp): This type of band was observed in Aa2, Aa3, Aa4, Aa5 and Aa6 isolates.

Band type 2 (1555 bp): This type of band was observed in Aa3 isolate.

Band type 3 (442 bp): This type of band was observed in Aa2, Aa3 and Aa4 isolates.

Band type 4 (205 bp): This type of band was observed in Aa1, Aa2, Aa3 and Aa4 isolates.

The banding pattern observed in primer OPA-10 is presented in Plate 10. The primer amplified in seven amplicons among six isolates of *Alternaria alternata.* The size of amplicons amplified ranges from 6003 to 123 bp. The polymorphism observed in this primer was 100 %. The details of the 7 RAPD band types are as follows.

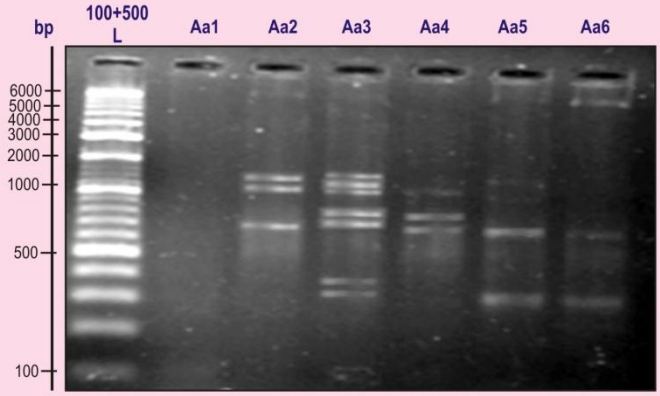

Plate 8: RAPD banding pattern of primer OPA-3

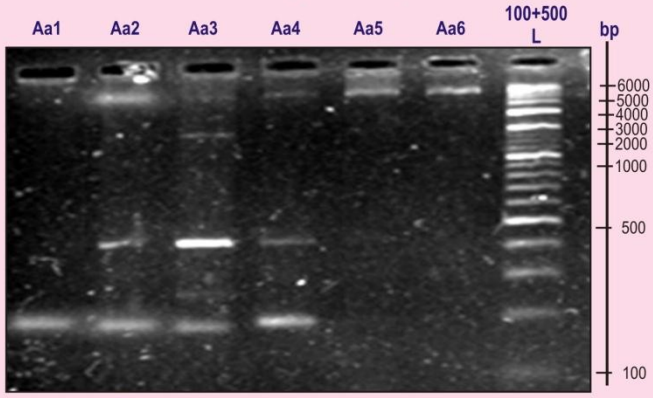

Plate 9: RAPD banding pattern of primer OPA-6

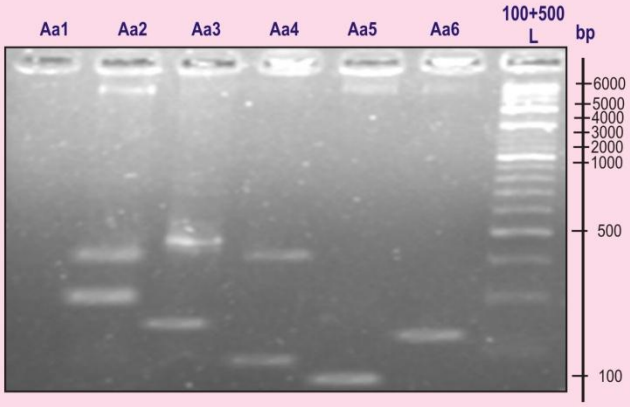

Plate 10: RAPD banding pattern of primer OPA-10

Band type 1 (6003 bp): This type of band was observed in Aa2, Aa3, Aa5 and Aa6 isolates.

Band type 2 (485 bp): This type of band was observed in Aa3 isolate.

Band type 3 (405 bp): This type of band was observed in Aa2 and Aa6 isolates.

Band type 4 (287 bp): This type of band was observed in Aa2 isolate.

Band type 5 (237 bp): This type of band was observed in Aa3 isolate.

Band type 6 (211 bp): This type of band was observed in Aa6 isolate.

Band type 7 (123 bp): This type of band was observed in Aa5 isolate.

The banding pattern observed in primer OPA-12 is presented in Plate 11. The primer amplified in seven amplicons among six isolates of *Alternaria alternata.* The size of amplicons amplified ranges from 6003 to 603 bp. The polymorphism observed in this primer was 100 %. The details of the 7 RAPD band types are as follows.

Band type 1 (6003 bp): This type of band was observed in Aa2, Aa3, Aa5 and Aa6 isolates.

Band type 2 (837 bp): This type of band was observed in Aa3 isolate.

Band type 3 (807 bp): This type of band was observed in Aa2 isolate.

Band type 4 (717 bp): This type of band was observed in Aa3 and Aa5 isolates.

Band type 5 (674 bp): This type of band was observed in Aa3 and Aa5 isolates.

Band type 6 (617 bp): This type of band was observed in Aa1 and Aa5 isolates.

Band type 7 (603bp): This type of band was observed in Aa2 and Aa3 isolates.

The banding pattern observed in primer OPA-13 is presented in Plate 12. The primer amplified in fifteen amplicons among six isolates of *Alternaria alternata.* The size of amplicons amplified ranges from 6009 to

237 bp. The polymorphism observed in this primer was 93.33 %. The details of the 15 RAPD band types are as follows.

Band type 1 (6009 bp): This type of band was observed in Aa1, Aa2, Aa3, Aa4, Aa5 and Aa6 isolates.

Band type 2 (2587 bp): This type of band was observed in Aa3 isolate.

Band type 3 (2204 bp): This type of band was observed in Aa2 isolate.

Band type 4 (2029 bp): This type of band was observed in Aa5 isolate.

Band type 5 (1999 bp): This type of band was observed in Aa3 and Aa4 isolates.

Band type 6 (1887 bp): This type of band was observed in Aa2, Aa4 and Aa5 isolates.

Band type 7 (1009 bp): This type of band was observed in Aa3 and Aa4 isolates.

Band type 8 (968 bp): This type of band was observed in Aa3 and Aa4 isolates.

Band type 9 (887 bp): This type of band was observed in Aa2 and Aa5 isolates.

Band type 10 (709 bp): This type of band was observed in Aa3 and Aa4 isolates.

Band type 11 (691 bp): This type of band was observed in Aa2 isolate.

Band type 12 (467 bp): This type of band was observed in Aa3 isolate.

Band type 13 (416 bp): This type of band was observed in Aa2 isolate.

Band type 14 (403 bp): This type of band was observed in type Aa3 and Aa4 isolates.

Band type 15 (237 bp): This type of band was observed in Aa3 isolate.

The banding pattern observed in primer OPA-14 is presented in Plate 13. The primer amplified in twelve amplicons among six isolates of *Alternaria alternata.* The size of amplicons amplified ranges from 6004 to 129 bp. The polymorphism observed in this primer was 93.33 %. The details of the 12 RAPD band types are as follows.

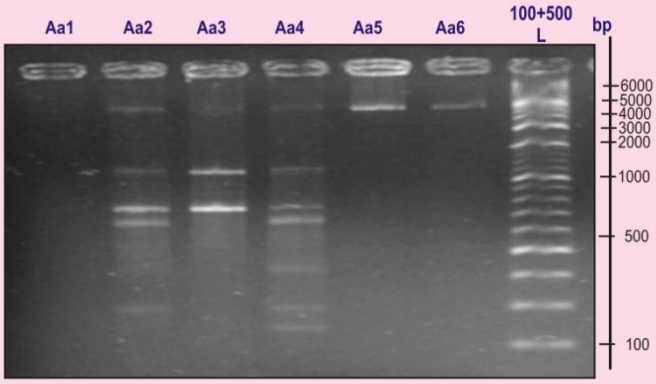

Plate 11: RAPD banding pattern of primer OPA-12

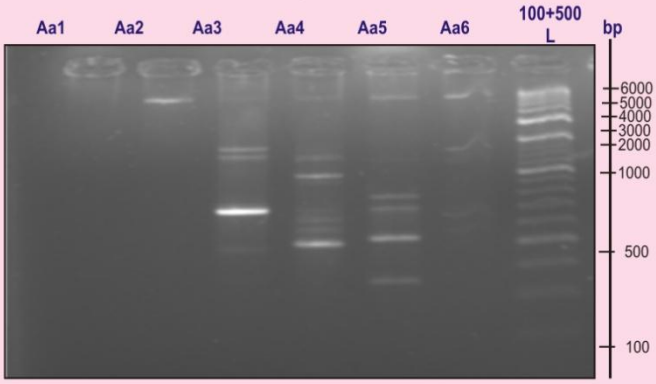

Plate 12: RAPD banding pattern of primer OPA-13

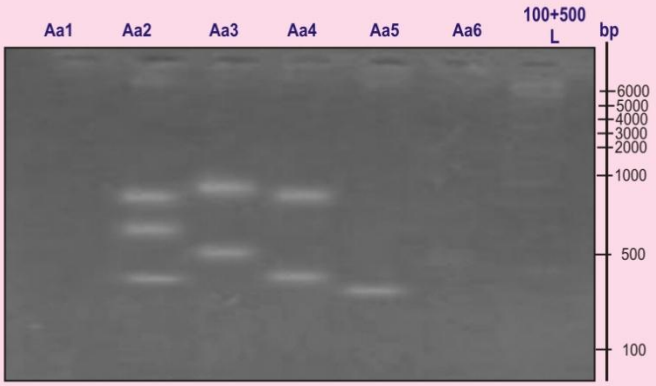

Plate 13: RAPD banding pattern of primer OPA-14

Band type 1 (6004 bp): This type of band was observed in Aa1, Aa2, Aa3, Aa4, Aa5 and Aa6 isolate.

Band type 2 (1909 bp): This type of band was observed in Aa2 isolate.

Band type 3 (1094 bp): This type of band was observed in Aa2 and Aa4 isolates.

Band type 4 (807 bp): This type of band was observed in Aa3 isolate.

Band type 5 (657 bp): This type of band was observed in Aa5 isolate.

Band type 6 (478 bp): This type of band was observed in Aa3 isolate.

Band type 7 (438 bp): This type of band was observed in Aa3 isolate.

Band type 8 (357 bp): This type of band was observed in Aa3 and Aa5 isolates.

Band type 9 (217 bp): This type of band was observed in Aa5 isolate.

Band type 10 (205 bp): This type of band was observed in Aa4 isolate.

Band type 11 (194 bp): This type of band was observed in Aa2 isolate.

Band type 12 (129 bp): This type of band was observed in Aa3 isolate.

The banding pattern observed in primer OPA-18 is presented in Plate 14. The primer amplified in seven amplicons among six isolates of *Alternaria alternata.* The size of amplicons amplified ranges from 5103 to 226 bp. The polymorphism observed in this primer was 93.33 %. The details of the 7 RAPD band types are as follows.

Band type 1 (5103 bp): This type of band was observed in Aa2, Aa3, Aa4, Aa5 and Aa6 isolates.

Band type 2 (1109 bp): This type of band was observed in Aa2, Aa3 and Aa4 isolates

Band type 3 (758 bp): This type of band was observed in Aa2, Aa3, Aa4 isolates.

Band type 4(687 bp): This type of band was observed in type Aa2 and Aa4 isolates.

Band type 5 (403 bp): This type of band was observed in Aa2 and Aa4 isolates.

Band type 6 (293 bp): This type of band was observed in Aa2 and Aa4 isolates.

Band type 7 (226 bp): This type of band was observed in Aa4 isolate.

The banding pattern observed in primer OPA-19 is presented in Plate 15. The primer amplified in eleven amplicons among six isolates of *Alternaria alternata.* The size of amplicons amplified ranges from 6009 to 227 bp. The polymorphism observed in this primer was 100 %. The details of the 11 RAPD band types are as follows.

Band type 1 (6009 bp): This type of band was observed in Aa2, Aa3, Aa4, Aa5 and Aa6 isolate.

Band type 2 (1909 bp): This type of band was observed in Aa3 and Aa6 isolates.

Band type 3 (1368 bp): This type of band was observed in Aa3 and Aa4 isolates.

Band type 4 (877 bp): This type of band was observed in Aa4 isolate.

Band type 5 (688 bp): This type of band was observed in Aa5 isolate.

Band type 6 (626 bp): This type of band was observed in Aa3 and 5 isolates.

Band type 7(507 bp): This type of band was observed in Aa4 isolate.

Band type 8 (486 bp): This type of band was observed in Aa5 isolate.

Band type 9 (411 bp): This type of band was observed in Aa4 isolate.

Band type 10 (379 bp): This type of band was observed in Aa3 isolate.

Band type 11 (227 bp): This type of band was observed in Aa5 isolate.

The banding pattern observed in primer OPB-11 is presented in Plate 16. The primer amplified in seven amplicons among six isolates of *Alternaria alternata.* The size of amplicons amplified ranges from 1479 to 194 bp. The polymorphism observed in this primer was 100 %. The details of the 7 RAPD band types are as follows.

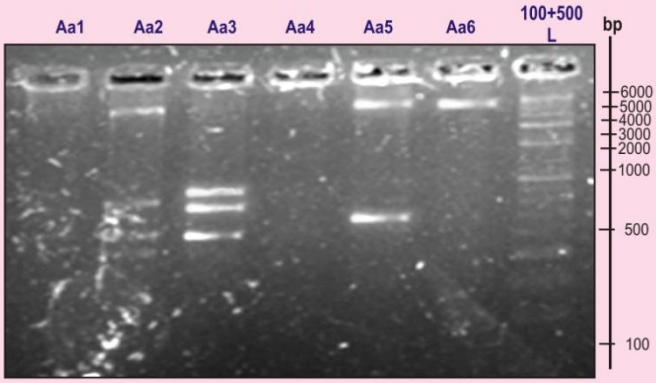

Plate 14: RAPD banding pattern of primer OPA-18

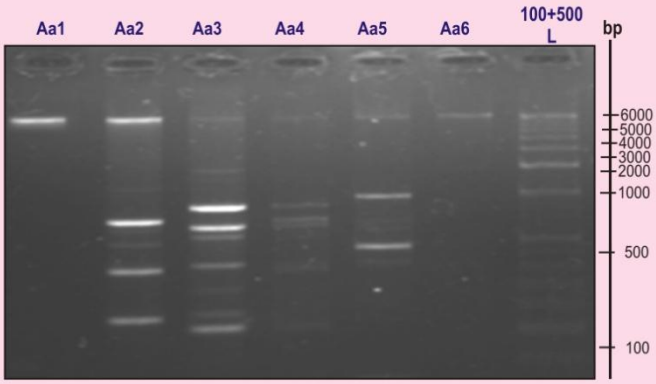

Plate 15: RAPD banding pattern of primer OPA-19

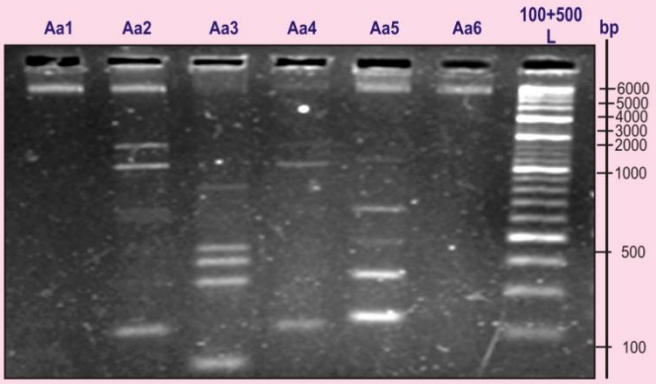

Plate 16: RAPD banding pattern of primer OPB-11

Band type 1 (1479 bp): This type of band was observed in Aa2, Aa5 and Aa6 isolates.

Band type 2 (547 bp): This type of band was observed in Aa3 isolate.

Band type 3 (507 bp): This type of band was observed in Aa2 and Aa4 isolates.

Band type 4 (377 bp): This type of band was observed in Aa2 isolate.

Band type 5 (327 bp): This type of band was observed in Aa3 isolate.

Band type 6 (227 bp): This type of band was observed in Aa2 and Aa4 isolate.

Band type 7 (194 bp): This type of band was observed in Aa5 isolates.

Binary similarity matrix for RAPD analysis:

A binary similarity matrix of combined data from 10 RAPD primers for 6 isolates of *Alternaria alternata* were prepared by scoring presence or absence of band. The of same molecular weight were assumed to be identical.

On the basis of calculated similarity matrix the similarity between genotypes can be predicted. The genotypes showing similarity index “1” are presumed to be 100% similar while that of “0” are 100% genetically dissimilar. In present study the similarity coefficient value ranged from 0.530 to 0.030 across six isolates of *Alternaria alternata* indicating high degree of polymorphism in respect to genetic similarity. Genetic similarity estimate (Jaccard’s coefficient) based on RAPD banding pattern was used for cluster analysis to present genetic relationship in the form of dendrogram. Jaccard’s coefficient value for six isolates are presented in Table 18.

In this Dendrogram higher value of similarity coefficient 0.536 was between Aa2 and Aa4, whereas 0.030 was between Aa1 and Aa2 found to have lower value of similarity coefficient. Two major clusters were obtained on the basis of analysis. First group is named as cluster –A includes Aa1. Second group is named as cluster –B which include Aa2, Aa4, Aa3, Aa5 and Aa6. The Aa2 was found to have a higher similarity

index with Aa4.The Aa1 with Aa2 and Aa1 with Aa4 was found to have a lower similarity index.

Table 18: Similarity coefficient for RAPD analysis

	Aa1	Aa2	Aa3	Aa4	Aa5	Aa6
Aa1	1.0000000					
Aa2	0.0303030	1.0000000				
Aa3	0.0689655	0.2978723	1.0000000			
Aa4	0.0312500	0.5365854	0.3043478	1.0000000		
Aa5	0.0400000	0.3658537	0.2619048	0.2500000	1.0000000	
Aa6	0.0625000	0.3428571	0.2222222	0.2432432	0.3928571	1.0000000

Shazia and Iftikar (2014) studied the genetic variation among the 17 isolates of *A*. *alternata* through RAPD primer. The all amplified bands were ranged in size from 400 bp to 2500 bp correlation produced between banding patterns. In a parallel study Ginoya and Gohel (2016) RAPD analysis of the eight isolates of *A. alternata* analyzed by 10 random primers produced 99 loci. Out of which, 98 loci were polymorphic. On an average, 98.98 per cent polymorphism was observed. Out of these, ten primers were found useful for amplification of DNA of *A. alternata.* Among the 10 primers, all primer gave 100% polymorphism of the DNA, which helped to ascertain variability except, OPF-1 primer having 85.71% polymorphism. Nasim et al. (2012) RAPD was used to determine the finger print of 10 isolates of *Alternaria alternata*. A total of 22 bands ranging in size from 250-1200 bp were generated by the primer GL-A-01. Nineteen of these 22 bands (86.36%) were monomorphic. Only three bands with sizes of 250, 600 and 1200 bp produced by primer GL-A-01, were polymorphic. Cluster analysis of similarity between band pairs showed that the fungal isolates were clustered in two distinctive groups.

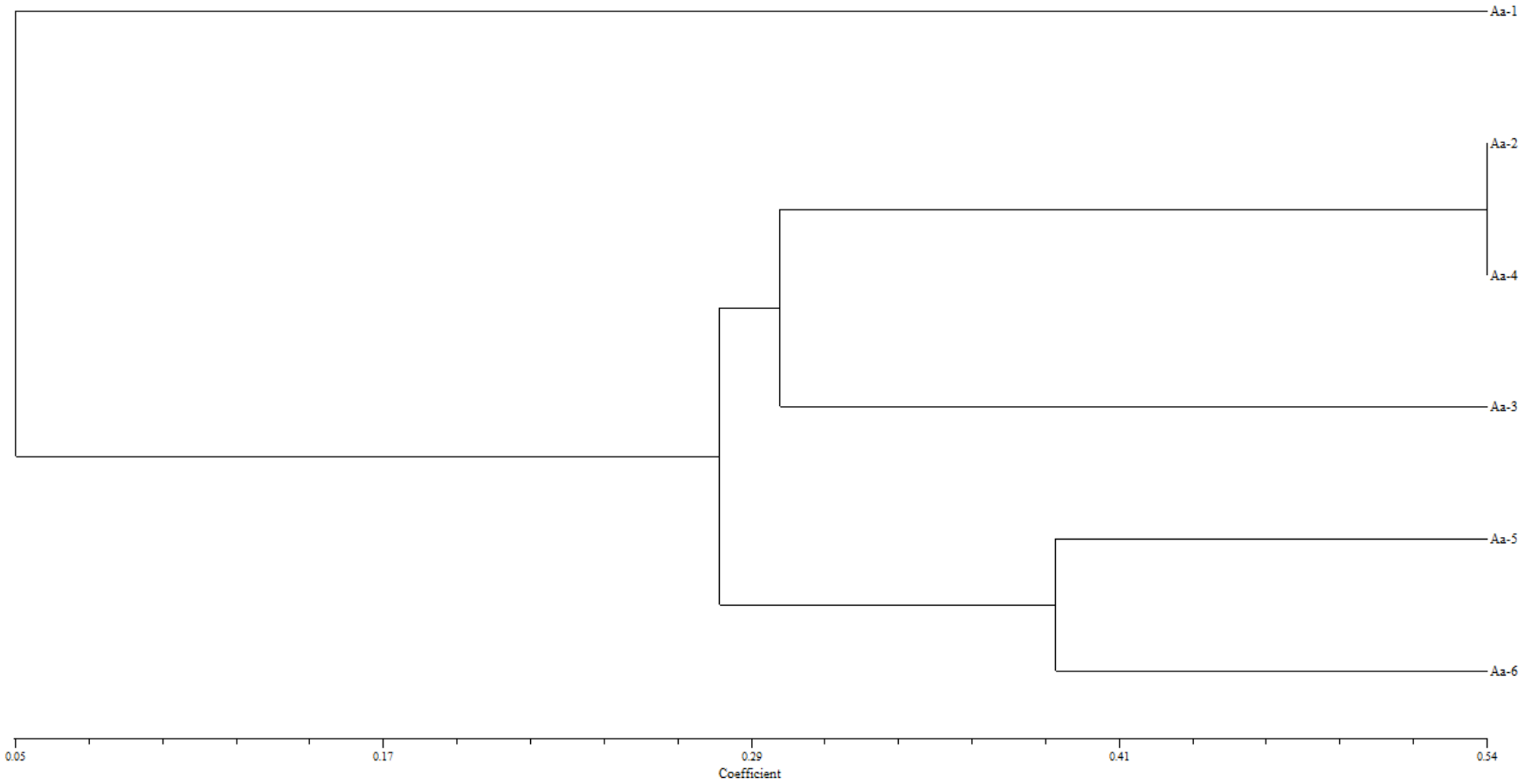

Fig. 1 The RAPD UPGMA dendrogram of 6 isolates of *Alternaria alternata* based on Jaccard's similarity coefficient

CHAPTER V

SUMMERY AND CONCLUSIONS

The investigation was carried out in the laboratory of the Department of Plant Pathology, Dr.P.D.K.V. Akola. The isolates selected for study of morphology and molecular variability of *Alternaria alternata* were Kolhapur (Aa1), Satara (Aa2), Sangli (Aa3), Akola (Aa4), Nagpur (Aa5) and Amravati (Aa6) collected from different chilli growing districts of Maharashtra.

Morphological variation

The isolates of *A. Alternata* selected for morphological studies showed variation on five different media *viz*. PDA, OMA, CDA, V-8 and CMA. The highest radial growth (75.65 mm) was recorded on Potato Dextrose Agar media. The second best medium was Oat Meal Agar media (59.4 mm). In case of isolates, maximum radial growth (53.50 mm) was recorded in Sangli isolate (Aa3) followed by Satara isolate (Aa2) i.e 52.5 mm. The conidial shape of *A. alternata* were found light to dark brown in colour with muriform, broadly rounded base formed in Aa1 and Aa4 isolates on PDA, OMA, CDA, V-8, while conidia are obclavate to oval in shape with blunt at tip Aa2, Aa3 and Aa5 isolates on all media. Maximum conidial length (43.30 µm) was noticed in CMA, followed by PDA (42.10 µm) in Aa1 isolate and maximum conidial breadth (12.4 µm) was noticed in PDA and OMA media in isolate Aa4 followed by Aa2 i.e 12.3 µm in PDA. The maximum number of transverse septa (7-9) were observed in Aa1 isolate on OMA media, while maximum (0-3) number of longitudinal septa were observed in Aa2 isolate on PDA.

In case of colony colour and colony margin isolates produced greenish to greenish brown colony colour with regular margin was observed in Aa1, Aa4, Aa2 isolates on PDA, OMA, CDA and CMA media. Whereas some isolates like Aa2 and Aa6 on PDA, OMA and CMA showed light gray to grayish black colony colour with regular to irregular margin. while Aa1 isolate produce grayish white pigmentation on V-8 media with irregular margin. Aa5 isolate showed light brown with white center

pigmentation with regular to irregular margin on PDA, OMA and CDA. While blackish with regular to irregular margin was observed in Aa5 and Aa3 on V-8 and CMA media. Aa1 and Aa4 isolate produce creamy white with regular to irregular margin on CDA, V-8 and CMA media.

All the isolates of *A*. *alternata* produce septate mycelium on five different media. Good sporulation was noticed in PDA media (2.8) having more than 25 spores in single microscopic field, while good sporulation 2.4 was recorded in Aa2 and Aa4 isolates.

Pathogenicity and symptoms

The ripe chilli fruit inoculated with *A. Alternata.* After 3-5 days of inoculation, formation of small necrotic lesions brown to black in colour and regular to irregular in shape, become sunken lesions. Isolate Aa5 was the most virulent isolate causing maximum lesion size of 5.5 mm with early initiation (3 days) followed by Aa6 and Aa1 which exhibited lesion size 4.1mm and 3.4mm, respectively. The lesion size recorded in Aa3, Aa2 and Aa4 are 2.5mm, 1.9mm and 1.1mm, respectively. Thus, *A*. *alternata* isolate Aa5 proved as the most virulent isolate in which maximum lesion size was observed.

Molecular variation

The molecular variability was studied among the six isolates of *A. Alternata* by using 15 RAPD primers of OPA and OPB series off which 10 primers produced 83 scorable bands. Among 83 bands 80 bands were polymorphic and level of polymorphism was 96.38%. The primer OPA-13 amplified maximum 15 bands within the size 6009 to 237 bp. While OPA-6 amplified minimum 4 bands within the size 5895 to 205 bp. The genetic similarity coefficient value ranged from 0.030 to 0.536 across six isolates of *A. Alternata*. Dendrogram showed higher value of similarity coefficient 0.536 was between Aa2 and Aa4, whereas 0.030 was between Aa1 and Aa2 found to have lower value of similarity coefficient. Two major clusters were obtained on the basis of analysis. First cluster is named as cluster –A, includes Aa1. Second group of cluster is named as cluster –B Aa2, Aa4, Aa3, Aa5 and Aa6. Respectively, For the confirmation of fungus

pathogen *A. Alternata* ITS marker was done with ITS-1 and ITS-4 primer which gives band size of 440bp.

Conclusions:

Six isolates of *A. Alternata* showed morphological variation among different media i.e PDA, OMA, CDA, V-8 and CMA. PDA and OMA was the excellent media to support the radial growth and sporulation of *A. Alternata.* Isolates produced muriform conidia with broadly rounded base to obclavate conidia blunt at the tip. Size of conidia ranging from 43.3-27.7×12.4-8.80 µm on different media. Septation among the isolates varied from 3-9 transverse and 0-3 longitudinal septa. Isolates produced greenish, grayish, brownish white and creamy white in colour with regular to irregular margin.

Pathogenicity test was done by using pin prick method. Aa5 and Aa6 was most virulent isolates whereas, Aa4 was the less virulent isolate.

The molecular variability was studied among the 6 isolates of *A. Alternata* by using 15 RAPD primers of OPA and OPB series off which 10 primers produced 83 scorable bands. Among the RAPD primer 83 bands 80 bands were polymorphic and level of polymorphism was 96.38%. Molecular diversity using RAPD marker showed that the Aa2 having higher similarity index with Aa4.

CHAPTER VI

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APPENDIX

Primer	Basepair	Aa-1	Aa-2	Aa-3	Aa-4	Aa-5	Aa-6
OPA-2	6009 bp	0	1	0	1	1	1
	1280 bp	0	1	0	1	0	0
	1197 bp	0	1	0	0	0	0
	490 bp	1	1	1	1	1	1
	410 bp	0	0	0	0	0	1
	344 bp	0	1	1	1	0	0
	260 bp	0	0	0	0	1	0
	207 bp	1	1	1	0	0	0
OPA-3	6005 bp	0	1	1	0	1	1
	1367 bp	0	1	1	0	1	0
	1280 bp	0	1	0	1	1	0
	780 bp	0	1	0	1	1	1
	395 bp	0	1	0	1	0	0
	345 bp	0	0	1	0	0	0
	326 bp	0	0	1	0	1	1
OPA-6	5895 bp	0	1	1	1	1	1
	1555 bp	0	0	1	0	0	0
	442 bp	0	1	1	1	0	0
	205 bp	1	1	1	1	0	0
OPA-10	6003 bp	0	1	1	0	1	1
	485 bp	0	0	1	0	0	0
	405 bp	0	1	0	0	0	1
	287 bp	0	1	0	0	0	0
	237 bp	0	0	1	0	0	0
	211 bp	0	0	0	0	0	1
	123 bp	0	0	0	0	1	0
OPA-12	6003 bp	0	1	1	0	1	1
	837 bp	0	1	0	0	0	0
	807 bp	0	1	0	0	0	0
	717 bp	0	0	1	0	1	0
	674 bp	0	0	1	0	1	0
	617 bp	1	0	0	0	1	0
	603 bp	0	1	1	0	0	0
OPA-13	6009 bp	1	1	1	1	1	1
	2587 bp	0	0	1	0	0	0
	2204 bp	0	1	0	0	0	0
	2029 bp	0	0	0	0	1	0
	1999 bp	0	0	1	1	0	0
	1887 bp	0	1	0	1	1	0
	1009 bp	0	1	0	1	0	0
	968 bp	0	0	1	1	0	0
	887 bp	0	1	0	0	1	0
	709 bp	0	0	1	1	0	0
	691 bp	0	1	0	0	0	0
	467 bp	0	0	1	0	0	0
	416 bp	0	1	0	0	0	0
	403 bp	0	0	1	1	0	0
	237 bp	0	0	1	0	0	0
OPA-14	6004 bp	1	1	1	1	1	1
	1909 bp	0	1	0	0	0	0
	1094 bp	0	1	0	1	0	0
	807 bp	0	0	1	0	0	0
	657 bp	0	0	0	0	1	0
	478 bp	0	0	1	0	0	0
	438 bp	0	0	1	0	0	0

	357 bp	0	0	1	0	1	0
	217 bp	0	0	0	0	1	0
	205 bp	0	0	0	1	0	0
	194 bp	0	1	0	0	0	0
	129 bp	0	0	1	0	0	0
OPA-18	5103 bp	0	1	1	1	1	1
	1109 bp	0	1	1	1	0	0
	758 bp	0	1	1	1	0	0
	687 bp	0	1	0	1	0	0
	403 bp	0	1	0	1	0	0
	293 bp	0	1	0	1	0	0
	226 bp	0	0	0	1	0	0
OPA-19	6009 bp	0	1	1	1	1	1
	1909 bp	0	0	1	0	0	1
	1368 bp	0	0	1	1	0	0
	877 bp	0	0	0	1	0	0
	688 bp	0	0	0	0	1	0
	626 bp	0	0	1	0	1	0
	507 bp	0	0	0	1	0	0
	486 bp	0	0	0	0	1	0
	411 bp	0	0	0	1	0	0
	379 bp	0	0	1	0	0	0
	227 bp	0	0	0	0	1	0
OPB-11	1479 bp	0	1	0	0	1	1
	547 bp	0	0	1	0	0	0
	507 bp	0	1	0	1	0	0
	377 bp	0	1	0	0	0	0
	327 bp	0	0	1	0	0	0
	227 bp	0	1	0	1	0	0
	194 bp	0	0	0	0	1	0