क्रूसीफर्स में कृष्णपर्ण धब्बा उत्पन्न करने वाले *आल्टरनेरिया ब्रैसिकी* (बर्क) सैक का हेतु विज्ञान एवं विविधता

Epidemiology and diversity of *Alternaria brassicae* (Berk.) Sacc the causal agent of black leaf spot in crucifers

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Epidemiology and diversity of *Alternaria brassicae* (Berk.) Sacc the causal agent of black leaf spot in crucifers

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This is to certify that the thesis entitled **"Epidemiology and diversity of *Alternaria brassicae* (Berk.) Sacc the causal agent of black leaf spot in crucifers"** submitted to the Faculty of the Post-Graduate School, Indian Agricultural Research Institute, New Delhi, in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy in Plant Pathology** is a record of *bona fide* research work carried out by **Mr. Selvamani, R. Roll No. 9727** under my guidance and supervision and that no part of this thesis has been submitted for any other degree or diploma. I further certify that any help or information received during the work on this thesis has been duly acknowledged.

Place: New Delhi

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(Pratibha Sharma)

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LIST OF ABBREVIATIONS

AB	*Alternaria brassicae*
BLAST	Basic local alignment search tool
BOD	Biological oxygen Demand
bp	base pair
ºC	Degree centigrade
cfu	Colony forming unit
DAS	Days after sowing
DAP	Days after planting
CRD	Complete randomized design
CPA	Carrot Potato Agar
dATP	Deoxyadenosine 5' triphosphate
dCTP	Deoxycytidine 5' triphosphate
dGTP	Deoxyguanosine 5' triphosphate
DNA	Deoxyribose nucleic acid
dNTP	Deoxynucleotide 5' triphosphate
dTTP	Deoxythymidine 5' triphosphate
EtBr	Ethidium bromide
ha	Hectare
hr	Hour
ISSR	Inter simple sequence repeat
ITS	Internal transcribed spacer
Kg	Kilogram
µg	Microgram
µl	Micro liter
ml	Milliliter
mha	Million hectare
mM	Millimolar
Mt	Million tone
M	Molar

min	Minutes
MSL	Mean sea level
NA	Nutrients agar
Ng	Nanogram
CLEA	Cauliflower leaf extract Agar
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PDB	Potato dextrose broth
RBD	Randomized block design
RAPD	Random Amplified Polymorphic DNA
Rpm	Revolutions per minute
RNA	Ribonucleic acid
RNase	Ribonuclease
Sec	Second
TBE	Tris borate EDTA buffer
U	Unit
UPGMA	Unweigheted pair group method using arithmetic averages

1. INTRODUCTION

Brassicaceae (crucifer) is an economically important family of flowering plants in the plant kingdom, the family contains about 3500 species and 350 genera, is one of the 10 most economically important plant families (Warwick *et al.,* 2000). It also has well-known species such as *Brassica oleracea* (broccoli, cabbage, cauliflower, etc.), *Brassica rapa* (turnip, Chinese cabbage,etc.), *Brassica napus* (rapeseed,etc.), *Raphanus sativus* (common radish), *Armoracia rusticana* (horseradish), Matthiola (stock), *Arabidopsis thaliana* (model organism) and many others.

Among these crops mustard, cauliflower and cabbage are cultivated widely. Among edible oilseed crops of India, mustard occupies important place along with soybean and groundnut. Rapeseed and mustard occupies 5.9 million ha in India, the total production being 6.8 million tonnes with an average yield of 1145 kg/ha in 2011-12 (Economic survey of India, 2013) and contributes around 23.7 per cent to the total oilseed acreage and 26.0 per cent of total oilseed production in India. Cabbage and cauliflower are also important winter vegetables in India. In India cabbage and cauliflower occupies about 3.69 lakh ha each, production accounts about 7.95 and 6.74 lakh ton respectively with the productivity of 21.5 and 18.3 Mt/ha respectively (Indian Horticulture Database by National Horticulture Board, 2011).

A number of biotic and abiotic stresses pose major constraints in mustard, cabbage and cauliflower production. Among the infectious disease black spot (*Alternaria brassicae, Alternaria brassicicola and Alternaria raphani*), black leg and phoma root rot (*Leptosphaeria maculans and Phoma lingam*), anthracnose (*Colletotrichum higginsianum*), black mold rot (*Rhizopus stolonifer*), white rust and staghead (*Albugo candida*) and black rot (*Xanthomonas campestris* pv. *campestris*) are important. The most common and destructive diseases of brassicaceae are those caused by four species of *Alternaria viz., Alternaria brassicae* (Berk.) Sacc., *Alternaria brassicicola* (Schwein.) wiltsh., *Alternaria raphani* Groves and Skolko, and *Alternaria alternata* (Fr.) Keissl. *Alternaria* blight caused by *Alternaria brassicae* (Berk.) Sacc. is the number one constraint in production of these crops (Kolte, 1985).

Berkeley (1836) identified the leaf spot disease and causal fungus as *Macrosporium brassicae* Berk., which was later renamed as *Alternaria brassicae* (Berk.) Sacc. by Saccardo

(1886). In India the disease was first reported by Butler (1918) in mustard and later the organism was identified as *Alternaria brassicae. Alternaria* has been reported from all continents of the world and in India also it was reported in most of the states. *Alternaria* blight appears every year in all the crucifer growing states of India, such as Rajasthan, Uttar Pradesh, Bihar, Odisha, West Bengal, Punjab, Haryana, Gujarat and Madhya Pradesh (Saharan, 1992a). *Alternaria* causes yield loss of up to 47per cent in mustard (Kolte, 1985), on cabbage, cauliflower, broccoli and other brassicas known to reduce the yield more than 50 per cent. The market losses are due to decay which develops in transit and storage. The *Alternaria* causes seed losses up to 50 per cent in cauliflower seed plants and from 70-90 per cent of harvest on rape and seed cabbage plantings (Kear *et al.,* 1977).

Leaf spot of *Alternaria brassicae* appears as small, dark coloured areas of necrotic lesions. They spread and form circular lesions up to 1.0 cm in diameter. In humid weather, dark conidiophores are seen on the surface of the lesion in concentric rings. Linear spots appear on petioles, stems and seed pods, which are often described as black and sooty with chlorotic yellow halos surrounding the lesion sites. *A. brassicae*, however, is not limited to infection of leaves, and can infect all parts of the plant including pods, seeds, and stems, and is of particular importance as a post-harvest disease. *A. brassicae* is considered a necrotrophic (causing eath) plant pathogenic fungus and like other *Alternaria* species has been shown to secrete numerous toxic secondary metabolites and proteins that cause cell death via induction of apoptosis in plants or by directly damaging cells. The *A. brassicae* causing disease on cruciferous plants can survive on or in crop debris, seed and possibly weed species in the crucifer family. Spores are produced on old crop debris and leaf spots. In culture, optimum temperatures for spore production are between 23° C to 27° C. However, if leaf wetness is prolonged for 20 hrs or more, *A. brassicae* is capable of producing many spores outside the optimum range of temperatures. Spore production at night or during long overcast periods is predominant. Spore production has been observed to be greater in yellowed leaves. Spores are released during the day as the relative humidity diminishes. Movement of equipment or work crews in the field and wind aids in spores release and spread. Spores have been trapped more than one and half kilometer down wind immediately after a crop with *Alternaria* leaf spot was harvested. After landing on susceptible plant tissue, spores of *Alternaria* are durable enough to remain there until free moisture in the form of dew, rain, or irrigation water occurs at which time the spore germinates. Penetration of

host tissue occurs without wounding the tissue. Infection of brassica leaves can occur between 27° C to 31° C.

In recent years, a great deal of interest has been generated in the study of variability using molecular tools, which makes the quantification of genetic variation as a relatively straight forward endeavor (Michelmore and Hulbert, 1987). Molecular tools provide exciting avenues for identification of pathogens and host genes. The use of DNA profiling systems reveals variation in the nucleotide sequence of DNA. The DNA based marker like Randomly Amplified Polymorphic DNA (RAPD), Inter Simple Sequence Repeats (ISSR), Simple Sequence Repeats (SSR), etc. are used widely to reveal the genetic variability among the pathogens. The biomolecule such as proteins, amino acids, and carbohydrates also plays significant role in host pathogen interactions. The biochemical markers like SDS-PAGE are also used for the diversity studies. The taxonomy of *Alternaria* is primarily based on morphological characteristics (Cho *et al.*, 2001), especially on differences in conidial morphology (Simmons, 1995). However, molecular approaches have been used increasingly in taxonomy and systematics of filamentous fungi. The rDNA genes have been employed to analyze major evolutionary events because it is highly conserved, whereas the rDNA internal transcribed spacer (ITS1 and ITS2) is more variable, and has been used for the investigation of species-level relationships (Bruns *et al.*, 1991). Thus the ITS region has been used in classifying fungi because of its systematic and taxonomic usefulness (Chillali *et al.*, 1998). This is because ITS regions are of suitable size for PCR amplification, restriction analysis and sequencing procedures, and because ITS regions are variable among species as well.

The absence of resistance genotype against *A. brassicae* and wide host range of *A. brassicae* has been great impediment in the management of the disease. The necessity to boost resistance breeding programme accentuates the need in depth study of the diversity of *A. brassicae* throughout crucifer growing areas in the country. The host pathogen interaction and cross compatibility becomes major issue in the management of *Alternaria*. One of the major challenges of research on crucifers is to develop technical knowledge about the epidemiological factors responsible for the development of *Alternaria* blight, ways to minimise the losses caused by these diseases and help stabilise mustard, cabbage and cauliflower production (Meena *et al.*,

2011). However, no effort has been reported so far regarding the use of AUDPC in relation to host pathogen interaction on different crucifers.

In India, very limited work has been done on *A. brassicae* in particular with molecular, biochemical variations and host pathogen interaction on different crucifers. Keeping in view, the economic importance of crucifers and reduction in its production due to *Alternaria* disease in India, the current research work has been undertaken with the following objectives:

Objectives

1. Role of weather on *Alternaria* leaf spot development in cabbage, cauliflower and mustard
2. Pathogenic variability and cross infectivity of *Alternaria brassicae* isolates.
3. Biochemical and molecular characterization of *Alternaria brassicae* isolates.

2. BACKGROUND

Alternaria blight caused by *Alternaria brassicae* (Berk.) Sacc. Is an important fungal problem that can severely affect the foliage and seed germination in crucifers. The information regarding *Alternaria brassicae* is limited in India and the world. The available literature on the various aspects of *Alternaria* spp. characterization including variability studies and epidemiology on the cruciferous crops is being reviewed and presented below.

The available literature has been reviewed under following sections:

2.1 History and occurrence

As early as 1836, Berkeley identified the causal fungus on plants belonging to the Brassicaceae as *Macrosporium brassicae* Berk., which was later renamed as *Alternaria brassicae* (Berk.) Sacc. by Saccardo (1886). In 1922, 1926 and 1945, respectively, Milbraith, Weimer and Rangel also described the same fungus. Then in 1947, Wiltshire separated the small and big spores forms as *Alternaria brassicicola* (Sch.) Wiltshire and *Alternaria brassicae* (Berk.) Sacc.

There are two species of *Alternaria* found on the species of brassica which are probably more confused than any other species in this genus (Wiltshire, 1947). The first is *Alternaria brassicae* (Berk.) Sacc. described as *Macrosporium brassicae* Berk. in 1836 and the second is the fungus commonly known as *A. circinans* (Berk. & Curt.) Bolle. or *A. oleracea* Milbraith or, incorrectly as *A. brassicae* (Berk.) Sacc. for which Wiltshire (1947) proposed the name *A. brassicicola* (Sch.).

Alternaria brassicae (Berk.) Sacc. and *A. brassicicola* (Sch.). have been reported from almost every continent on Brassicaceae hosts. *A. brassicae* is considered to be most destructive on oil yielding brassicas and both are common on vegetable crucifers. They are known to occur in Argentina, Australia, Bangladesh, Brazil, Britain, Bhutan, Bulgaria, Myanmar, Canada, Chile, China, Cyprus, Czech republic, Denmark, England, Ethiopia, Finland, France, Germany, Ghana, Hungary, India, Ireland, Italy, Jamaica, Japan, Kenya, Libya, Malawi, Malaysia, Morocco, Mozambique, Nepal, Netherland, New guinea, New Zealand, Nicaragua, Nigeria, Norway, Pakistan, Philippines, Poland, Romania, Russia, Saudi Arabia, Singapore, Spain, South

Africa, Sri Lanka, Sudan, Sweden, Thailand, Turkey, USA, etc. In India it is prevalent in all crucifer growing states.

2.1 Symptomatology

Symptoms of the disease are characterized by formation of spots on leaves, stem and silique. Leaf spots of *Alternaria brassicae* appear as small, dark coloured areas. They spread and form circular lesions up to 1.0 cm in diameter. On seedlings, symptoms include dark stem lesions immediately after germination that can result in damping-off, or stunted seedlings. In humid weather, dark conidiospores are seen on the surface of the lesion in concentric rings. Linear spots appear on petioles, stem and seed pods. In general, lesions produced by *A. brassicae* are grey compared to black sooty velvety lesions produced by *A. brassicicola* (Tahvonen, 1979). With progress, the disease appears on middle and upper leaves with smaller sized spots, when defoliation of lower leaves occurs. Later, round black conspicuous spots appear on silique and stem. Spots on mustard silique are brownish black with a distinct grey centre. The infection of Alternaria blight on leaves and silique reduces the photosynthetic area drastically. A brown discoloration of the cauliflower a curd occurs which darkens to an olivaceous colour with age. *Alternaria brassicae* attacks cabbage heads mostly after harvest. In long storage they overrun the outer leaves and sporulates profusely, giving a black, moldy appearance. In this phase, they commonly associated with the incidents of Rhizopus soft rot and grey mold rot (Chupp and Sherf, 1960).

2.3 Losses caused by the disease

Alternaria leaf spot is one of the severe diseases of mustard and vegetable crucifers affecting crops as well as seed crops. Heavy infestation on leaves, stems, and silique often influence both quantity and quality of yield of brassica crops (**Fig. 2.1**) (Butler, 1918). In India, yield losses in *Alternaria* infected plants increase considerably after winter rains (Dey, 1948). Shriveling of seed and reduction in quantity of oil content is the major effect in severe infections (Chahal and Kang, 1979; Chohan, 1978; Kaushik *et al*., 1984, Vasudeva, 1958). The seed production of brassicas has been reported to be greatly reduced by the attack of this disease which invade silique and penetrate the seeds besides damaging the assimilatory tissues of the leaves and stem (Bandhopadhyay *et al*., 1974; Nielson, 1933). Alternaria brassicae infection is also known to

Fig. 2.1 Crucifer field infected with Alternaria blight
(a) Mustard and Cabbage field (b) Cauliflower field

affect chemical composition of seed including protein, total carbohydrates and ash (Dengenhardt *et al.*, 1974; Nijhawan and Hussain, 1964).

In vegetable crops, losses occur from damping off of seedling and spotting of lower leaves and heads of cabbage. The disease can be destructive in seedbeds, especially in cabbage and cauliflower. Spotting and browning of cauliflower and cabbage heads reduce quality and market value of these crops (Sherf and Macnab, 1986). Reduction in seed yield may be high as 80% and the fungus may severely depress germination to the extent that infected seed may be unsalable (Smith *et al.* 1988). This disease had recently been recognized as an important cause of deterioration of white cabbage in cold storage (Kear *et al*., 1977). According to Gorshkov (1976), damping-off of cabbage caused by *A. brassicae* may result into 80-100 per cent losses. In wet seasons in the USA, *A. brassicae* leaf spot on cabbage, cauliflower, broccoli and other brassicas has been known to reduce the yield by more than 50%. The market losses are due to decay which develops in transit and storage (Ramsey and Smith, 1961). In general in India, losses of 15 to 71% were reported in all crucifers (Kadian and Saharan 1983; Singh and Bhowmik 1985, Kumar 1986).

2.4 Disease Cycle

The primary infection results by the wind borne spores produced on debris of previous season crops, weeds and other collateral host. Seed is also act as primary sources especially in temperate regions. Conidia readily germinate in the presence of moisture by giving rise to a germ tube which emerges from any cell of the spores. Theoretically only one spore can cause infection in the brassicaceous seedling indicating a threshold infection value at a very low level (Czyzewska, 1971). Germ tubes from germinated spores of *A. brassicae, A. brassicicola, A.raphani* and *A. alternata* generally penetrate the undamaged tissues of many brassicaceous hosts directly (Czyzewska, 1971; Changsri and Weber, 1963), although indirect penetration through stomata has been reported *A. brassicae* (Changsri, 1961; Tsuneda and Skoropad, 1978)

Alternaria conidia are thought of as “dry conidia” which are wind dispersed, maximum release taking place in the mid-afternoon. There is no report on the numerical threshold for infection; consequently lesions may be initiated from a single germinating conidium. Maude and Humpherson-Jones (1977) found that infected marrow stem kale (*B.oleracea var. acephala*) liberates 50 spores per cubic meter of air just prior to harvesting, 190 spores per cubic meter of

air at cutting, and 3200 spores per cubic meter of air when windrowed crops are harvested. Spores can be carried up to 1000 m downwind during harvesting. Frequent infection occurs when water is present on the host surface due to dew formation.

With *A. brassicicola* cuticular penetration is most frequent and stomatal penetration occurs to a lesser extent. Penetration is preceded by formation of appresoria from the tip of germ tubes and are either spherical or club-shaped and 3-8 mm in diameter. Stomatal penetration is more common with *A. brassicae*. Once within the host, the epidermal cells are fully invaded and mycelia ramify through and between the mesophyll and palisade cells, the entire leaf is soon parasitized. Early in the post-penetration phase, the invaded epidermal cells become necrotic, and parenchyma tissues ahead of the advancing hyphae often collapse (Dixon, 1981).

On the leaves of *B. napus* cultivar midas and *B. rapa* cultivar Torch conidia of *A. brassicae* germinate at the rate of 12.1% and 19.5% respectively, at 9 hr after inoculation. Conidia usually germinate by producing either germ tubes or secondary conidia. Penetration of leaves by *A. brassicae* is abundant at 24 hr and occurs either with or without the formation of appressoria. Penetration of the cultivar Torch leaves occurs either directly through epidermal cells or indirectly through stomata, while Midas leaves are penetrated almost exclusively through the stomata. Black spot lesions develop within 48 hr after inoculation (Tsunedo and Skoropad, 1978b). According to Tewari, (1983, 1986), *A. brassicae* in rapeseed becomes subcuticular after direct penetration. This is followed by colonization of the epidermal and the mesophyll cells. In the leaves of rapeseed cultivars Candle and Altex the pathogen heavily colonizes the necrotic centre and is not present in the chlorotic area indicating that a diffusible metabolite may be directly or indirectly responsible for leaf chlorosis. The plasma membrane is the first target of the diffusible metabolite. Subsequently, the chloroplast is either directly or indirectly affected leading to leaf chlorosis. Relative to chloroplast, the effect on mitochondria is seen at a much later stage. The cells in the necrotic area are almost completely devoid of cellular organelles and reveal electron dense lamellar deposites (Tewari, 1983). *Alternaria* infection in the *C. abyssinica* leaves is preceded by toxic substances secreted by the fungus. The same may be true for other Brassicas. According to Suri and Mandahar (1984; 1985), cytokinin like substances appears to be actively involved in the infection and pathogenesis of *A. brassicicola*. Production of cellulases and pectolytic enzymes by *A. brassica* causing leaf blight disease of rapeseed mustard has been observed (Suri and Mandahar, 1982). The differential susceptibility of the Brassicaceous host to

A. brassicae, A. brassicicola and *A. raphani* has been correlated with differences in amino acids content (Changsri, 1961).

The population of the pathogen varies with the age and variety of the host, maturity of the leaves and climatic conditions. Both *A. brassicae* and *A. brassicicola* occur in greater profusion on the leaves of *B. rapa* var. Yellow sarson then on the leaves of *Eruca sativa.* The younger leaves have lower incidence of disease of either pathogen compared to older leaves. Spores remain intact on susceptible plants until moisture from dew or rain allows them to penetrate into the tissue and cause a lesion. These lesions produce further spores and infections can then be spread throughout the crop by either the wind or rain (Sharma *et al.,* 1986).

A. brassicae and *A. brassicicola* also survive in the form of microsclerotia and chlamydospores which appear after infected leaves have partially decayed (Tripathi and Kaushik, 1984). Microsclerotia and chlamydspores of both pathogens can be formed within conidial cells. Both microsclerotia and chlamydospores develop best at low temperatures (3°C) and are resistant to freezing and desiccation (in vitro studies). Chlamydospores also can develop in conidial cells on natural soil at room temperature (Tsuneda and Skoropad, 1977).

2.5 Disease development in relation to environmental components

Information on influence of weather factors on development of disease can help in further refining disease management strategies (Dang *et al.,* 1995; Singh *et al.*, 1998). Alternaria blight of oilseed brassicas caused by *A. brassicae* is favoured by low temperature, high humidity and splashing rain (Humpherson-Jones and Phelps, 1989). The disease intensity increases with temperatures and relative humidity of the field are 21.5^{o}C and 85% respectively. In India, a temperature range of 15-25°C, relative humidity of 70-90%, intermediate winter rains and wind velocity around 2-5 km per hr has been reported to be most conducive to Alternaria blight development in mustard (Ansari *et al.,* 1989; Saharan, 1991; Saharan and Kadian, 1984; Saharan *et al.,* 1981). Sharma and Gupta (1978) trapped *A. brassicae* and *A. brassicicola* spores from the air during October to April on brown sarson crop fields under Indian conditions. Conidia of *A. brassicae* were intercepted at all hours of exposure but conidia of *A. brassicicola* were seen only for a specific period during the day. Maximum conidial catch was observed in March which coincides with the maximum manifestation of disease in the field. The infection processes of both *A. brassicae* and *A. brassicicola* are initiated if water is present for 5-8 hr. For infection of *A. brassicae* in cauliflower leaves and heads, 25-31°C temperature is reported as optimum

(Weimer, 1924); the disease increases with the increase in humidity of the surrounding air. In rapeseed, *A. brassicae* establishes itself in less than 6 hr at 22°C (Louvet and Billotte, 1964). For infection of cabbage plants, both *A. brassicae* and *A. brassicicola* require free water with an optimum temperature of 25°C for *A. brassicicola*, and 15°C for *A. brassicae.* At these temperatures a minimum of 16 hr is necessary for the initiation of infection, and 48-72 hr for optimal disease development. Alternating wet and dry (70-80% RH) periods of 16 hr and 18 hr respectively restrict infection of both species. The differential temperature requirements of the two species become most marked at 10°C and 96 hr of incubation after inoculation; *A. brassicicola* fails to produce significant infection, whereas infection by *A. brassicae* produces numerous lesions (Humpherson-Jones and Hocart, 1983).

Current information about epidemiological factors in relation to disease development and predication of Alternaria blight of rapeseed-mustard had been studied by Awasthi and Kolte (1994) and they reported that total rainfall and minimum temperature had a significant contribution in the MRA equation and the combined effect of RH, rainfall and minimum temperature accounted for >78% contribute for prediction of *A. brassicae* severity on leaves of both crops.

2.6 Models to describe the progress of the disease

Several models have been proposed to describe the progress of compound interest disease like *Alternaria* disease of crucifers (Fontem *et al*., 1991):

i. Gompertz equations : Y= 1n [-1n(Y)]

ii. Logistic equations : Y= 1n[y/(1-y)]

To illustrate some possibilities, Fontem *et al.* (1991) evaluated progress and spread of dark leaf spot in three cabbage cultivars during two seasons by fitting representative curves of eight gradient models. The progress of the disease among cultivars is compared with observed initials disease (Y), epidemic rate (K), final disease severity (yf) and the area under the disease progress curve (AUDPC) (Shaner and Finney, 1977). The volume under the isopathic rate C rate of movement in space of a given level of disease severity (Berger and Luke, 1979) are calculated from the progress and spread characteristics of the disease. The VUDPS (volume under disease progress surface) is calculated from the AUDPC at each point in space as

VUDPS = Σ{ [A1 + 1+A2/2] [xi + 1 –xi]}

Where, A1 = the AUDPC at xi units of distance from the source. The isopathic rates are calculated from the source to 6.7 m for the y=0.1 isopath in the winter seasons and y=0.05 isopath in the spring season.

Meena *et al.,* 2005 used the AUDPC to compare the disease progression in different mustard varieties, which were shown on different dates. In the same study the Apparent Infection rates (r) was calculated. The apparent infection rates were further used to assess the highest and least infection periods with respect to each crop cultivars in relation to age. Kumar and Kolte (2001) have used the area under disease progress curve (AUDPC) to identify cultivars demonstrating a slow blight progress compared to others.

Meena *et al.*, 2002 reported that date of sowing had a major influence on the incidence of disease in mustard crops. Severity of leaf blight caused by *A. brassicae* and *A. brassicicola* increased with delay in sowing, being lowest on October sown crops and highest on December planted ones (Mian and Akanda 1989).

Van der plank (1975) first suggests that yield loss may be related to the AUDPC and demonstrated a linear relationship. In India for mustard Kolte *et al.,* 1987 used the AUDPC for yield loss assessment.

2.7 Sporulation

(a) Culture media:

A number of culture media have been reported suitable for growth and sporulation of *Alternaria* species pathogenic on brassicaceous plants. According to Neergaard (1945), *A. brassicae* sporulates profusely on malt extract and standard nutrient agar, but very poorly on PDA. Atkinson (1950), on the other hand, found good sporulation on both PDA and malt agar. According to Ansari *et al.* (1988), although *A. brassicae* grows and sporulates well on a wide range of media, PDA was found to be the best. Changsri (1960, 1961) found slightly poorer growth of *A. brassicae* in comparison to *A. brassicicola* and *A. raphani* which grow well on a wide range of agar media. On PDA, Mukadam and Deshpande (1977) found that *A. brassicae* not only grew and sporulated poorly, it also lost its ability to grow and sporulate with successive subculturing. Single spore transfers of *A. brassicae* on 10% alfalfa decotion agar produces the largest amount of spores (McDonald, 1959). It grows well on pechay decotion agar, corn meal agar, Czapek’s agar, coconut agar, leonian agar, PDA, V-8 juice agar, oat meal agar, nutrient

agar and prune agar, while sporulation is abundant on all but corn and oat meal agar (Lapis and Ricaforte, 1974). Billotte (1963) induced abundant sporulation of *A. brassicae* by the slow desiccation of culture in open petri dishes preceded by removal of the aerial mycelium and washing in running water. According to Degenhardt (1973), V-8 juice agar with Rose Bengal plus streptomycin stimulates sporulation of both *A. brassicae* and *A. raphani*

(b) Temperature and Relative humidity:

The optimum temperature for maximum sporulation of *A. brassicae* and *A. raphani* is between 23-25°C (Singh, 1980). *A. brassicae* have more demanding growth requirements, with the distinct optimal growth peak at 22.5^{o}C (Gupta *et al.* 1972). The temperature growth optimum of *A. brassicicola* is 25-27°C, but the growth continues to the extremes of 6°C and 37°C. According to Changsri (1960) and Changsri and Webber (1960), the optimum temperature for growth in culture of *A. brassicicola, A. brassicae* and *A. raphani* are 24-28°C, 20-24°C and 24-28°C respectively. However according to Taber *et al*. (1968) *A. raphani* and *A. brassicae* grow better between 20-25°C on malt agar. Lapis and Ricaforte (1974) report profuse mycelial growth and sporulation of *A. brassicae* at 16 to 24°C, but according to Ansari *et al.* (1989), the optimum is 23°C.

(c) Hydrogen ion concentration: The optimum pH requirement for growth and sporulation of *A. brassicicola, A. brassicae* and *A. raphani* are 6.0 to 8.0, 7.1 to 8.0 and 7.1 to 8.0 respectively (Gupta *et al.* 1969). According to Gupta *et al*. (1969) *A. brassicae* isolated from *B. oleracea* var. *botrytis* tolerates a wide pH range from 3.0 to 9.0, the optimum being 5.5. No sporulation occurs at pH level below 3 and above 9. Very good sporulation occurs at pH levels between 5.0 to 6.5.

(d) Light and darkness: Maximum growth and sporulation of *A. brassicae* occurs with alternating light and darkness. Continuous light completely inhibits sporulation. In light a definite zonation of spore production occurs (Ansari *et al*. 1989). According to Sasaki *et al.* (1985), sporulation of *A. brassicae* was inhibited by monochromatic radiation between 350 to 620 nm and reduced by continuous irradiation of UV radiation shorter than 350 nm.

2.8 Spore germination

Gupta *et al* (2003) found that there is a negative correlation with the temperature and positive correlation with relative humidity. According to the Degenhardt *et al.,* (1982) Germination requires the presence of moisture in the form of free water or high relative humidity (at least 95%).

(a) Effect of culture media:

The germinability of spores of *A. brassicae* and *A. brassicicola* is higher when cultivated on rich media than the spores produced on complex or poorly nutritive media. The presence of metabolic inhibitors in the growth media reduces spore germinability even in the presence of nutrients (Czapek's-Dox agar) and causes abnormalities in their morphology. Spores of both the species show maximum germinability irrespective of their age (up to 20 days). Increase in the age of the spores (30 days onwards) either increases their latent period and/or reduces their germinability, even in the presence of nutrients (Gupta *et al.,* 1969).

(b) Effect of temperature and relative humidity:

In *Alternaria* brassicae causing brown rot of cauliflower the optimum temperature for spore germination is 32-35°C with the minimum of <1.5°C and the maximum of 40-46°C; as no temperature between 40.5°C and 46°C was tried, the exact maximum temperature was not determined. The optimum temperature for mycelial growth is 25-27°C (Weimer, 1924). Degenhardt *et al.* (1982) observed excellent germination of *A. brassicae* spores at 15°C with 90% relative humidity and 6-8 hours of host leaf wetness period in contrast to very poor germination of *A. brassicicola* spores under similar conditions. Gupta *et al.* (1972) found 17-19°C to be optimal for conidial germination of *A. brassicae*. According to Ansari et al. (1988b), *A. brassicae* spores germinate over a very wide range of 10-28°C with an optimum of 23°C. However, Kadian and Saharan (1984) recorded 25°C as the optimum temperature for spore germination of *A. brassicae*. Spore germination commences after 4 hr and reaches its peak after about 24 hr relative humidity of > 90% is essential for maximum spore germination. Conidial germination of *A. brassicicola* is optimal at 30°C (Gupta *et al.,* 1972). Sarkar and Sengupta (1978) observed that conidia of *A. brassicicola* germinate best at 90-100% relative humidity and a temperature of 22-32°C. Self-inhibition of *A. brassicicola* occurs when the spore load in the germination medium is 0.2 optical density. The self-inhibiting compound is volatile at 35°C and denatures at 90°C, and is thought to be a substance of low molecular weight (Mukadam, 1982). Barton and Fine (1958) obtained 86-90% spore germination of *A. brassicicola* in the presence of 200 ppm gibberellic acid. Conidia of *A. alternata* causing leaf and pod blight of radish germinate at 15-30°C with an optimum at 20-25°C (Singh and Suhag, 1983).

(c) Effect of Host Extract and Exudates:

Leaf extracts of Brassica spp. are generally inhibitory to spore germination of *A. brassicae*. Leaf extracts and exudates of both susceptible and resistant cultivars are inhibitory but inhibitory effects of resistant cultivars like Tower and RC781 are more pronounced; generally, leaf exudates are more inhibitory than leaf extracts (Kadian and Saharan, 1984). The effect of leaf exudates of yellow sarson (*B. rapa*) and taramira (*E. sativa*) is reported to vary with the host variety, age of host plant and maturity of the leaves (Sharma *et al.*, 1985).

(d) Effect of light intensity:

Direct exposure to strong light intensity is deleterious to A. brassicae spores, inhibiting germination and reducing host infection (Kadian and Saharan, 1984). Maximum sporulation of *A. brassicae* occurs with alternating light and darkness. Continuous light completely inhibits sporulation (Ansari *et al.,* 1989; Changsri and Weber, 1963; Gupta *et al.,* 1972; Mridha, 1986; Mukadam and Deshpande, 1979; Sasaki *et al.,* 1985; Taber, 1964).

2.9 Cultural variation

Variations in cultural characteristics and pathogenesis of different isolates of four *Alternaria* species infecting Brassicaceae have been observed. However information on existence of distinct pathotypes using stand host differentials is rather limited (Saharan, 1992). Preliminary reports on variability in *Alternaria brassicae* were made from Holland and United Kingdom (Mridha, 1983). Similarly isolates of *A. brassicae* obtained from rapeseed showed differences in cultural growth on cherry agar and differed in their pathogenesis on seedlings.

Ramegowda and Naik (2008) reported that 14 isolates of *Alternaria* spp. showed light grey to blackish colour, and among 14 isolates six isolates showed irregular margin where as another set of eight isolates colony with smooth margin on PDA medium. The maximum radial growth was noticed when incubated in alternate dark or light cycles of 12 h each (85 mm). The cultural characteristics such as growth behavior and colony character were studied by Singh *et al*., 2007 and describe the isolates having lot of morphological and cultural variation. In similar way Goyal *et al*., 2011 observed variation in mycelial growth and sporulation among *A. brassicae* isolates

Prasad *et al*. (2009) studied the 26 isolates of *A. helianthi* in India and grouped them in to four based on the culture shape, pigmentation and colony growth on PDA medium. Authors mentioned that 11 isolates of *A. solani* radial growth on PDA medium at ±25ºC ranged from 14.9 mm to 57.7 mm. Growth of most of the isolates was not significantly different among three

different selective media, while radial growth recorded on PDA was significantly different (Kumar *et al*., 2008).

2.10 Morphological variation

Simmons (1992) organized the genus into 14 species groups based upon characteristics of conidia and chain formation. In subsequent work with *Alternaria* isolates recovered from pear. Simmons and Roberts (1993) further advanced the species group concept by referring to certain groups using a representative species, for instance, the alternata group, the tenuissima group and the infectoria group. Additional species groups discussed in other work include the arborescens, brassicicola, porri and radicina groups (Pryor and Gilbertson, 2000; Roberts *et al*., 2000; Simmons, 1995; Simmons and Roberts, 1993).

Pryor and Michailides (2002) studied the morphological characters of five *Alternaria* isolates of pistachio and isolates were grouped as identical or very similar to either *A. alternata*, *A. tenuissima*, *A. arborescens* or *A. infectoria*. Most of the *Alternaria* sp. exhibit considerable morphological plasticity that is dependent upon cultural conditions of substrate, temperature, light and humidity (Misaghi *et al*., 1978; Nishimura and Kohmato, 1983). Ellis (1971) reported that the conidia of *A. solani*, the causal agent of early blight on tomato are dark muriform, pale golden or olivaceous brown, smooth and usually 150–300 µm in length and 15–19 µm thick in the broadest part, with 9–11 transverse septa and 1–4 longitudinal or oblique septa; sometimes branched 2.5–5 lm thick tapering gradually.

Goyal *et al*., 2011 studied the morphological variations among the *A. brassicae* isolates by using conidial length, conidial width, beak length and number of septa in conidia and found that average conidial length was 31.2 to 51.8 µm, average conidial width varied from 6.7 to 9.6 µm, average beak varied from 8.2 to 20.6 µm, the average transverse septa varied from 0 to 0.4. The microscopic examination revealed variability in conidial morphology and they characterized into two groups.

2.11 Pathogenic variation

The knowledge on variability in respect of virulence and aggressiveness of a pathogen is considered to be an important aspect for disease management. Pathogen aggressiveness is defined as the relative ability of a pathogen to colonize the host and cause damage and virulence as the relative capacity to produce disease (Shurtleff and Averre, 1997). The pathogenic

variability of *Alternaria alternata* infecting senna was studied by Tetarwal *et al*. 2008, and noted the significant variation. Study also reveals that the isolates virulence pattern.

Vishwanath and Kolte (1997) studied variability *Alternaria brassicae* in response to different host and found the significant variability among the isolates in different host in terms of symptom development and disease development. In another studies the same author found the biochemical variation among *Alternaria brassicae* isolates in terms of contents of biochemicals like proteins, lipids, etc. Awasthi and Kolte (1989) distinguished three isolates A, C and D from Pantnagar on the basis of pathogenesis. Kolte *et al.* (1991) further indicated that Pantnagar isolates resembled Bihar and Kanpur isolates. Viswanath and Kolte (1997) further suggested that isolate A was more virulent, C was moderate and D was avirulent on the basis of their reaction on 14 host genotypes in five different *Brassica* species. Wide variations in virulence among the races of *A. brassicae* have also been observed by Saharan and Kadian (1983) through studies were based on isolates collected from the same location. Hong *et al.*, 1996 differentiated the isolates of *A. brassicae* on the basis of their virulence. They concluded that 53 strains could be divided into 5 types of virulence as AB1, AB2, AB3, AB4 and AB5 ranging from low to high virulence.

Mehta *et al*., 2003 studied the morphological and pathological variability in *A. brassicae* in rapeseed and mustard. In this experiment he used fifteen host differentials in different Brassica species and found that each isolate behaved differently in host differential, the comparative study revealed that all the differential were susceptible to different isolates of *A. brassicae* and the range of infection period varied from 3-13 days in isolates but in general, majority took 3-5 days to cause the infection. Pathogenic diversity of isolates of *A. brassicae* were studied by Kumar *et al*., 2003, all those isolates were tested on a set of seventeen host differentials of different species of brassicae in green house conditions and results revealed that all those isolates behaved differently in host differentials. The studies indicated the existence of variability among isolates of *A. brassicae* in Haryana.

Incubation and latent period exhibited greater variability and minimum incubation period of three days were observed. *Alternaria* isolates differed significantly for their pathogenicity on different wild *Helianthus* species. Twenty six isolates were categorized into three pathogenicity groups based on disease incidence recorded on different *Helianthus* species as low (< 20% PDI), medium (20-50% PDI) and high pathogenicity (> 50% PDI) groups (Prasad *et al*., 2007).

2.11 Biochemical variation

Electrophoresis is the choicest method to resolve protein polymorphism. Polyacrylamide gel electrophoresis was introduced by Raymand and Weintraub (1959). A simple vertical slab gel electrophoresis was constructed by Beileski and Reid (1968). This technique is commonly used in developing genetic fingerprinting of crop varieties, inbred lines and hybrids. Apart from these advantages, it has some limitations such as all genetic changes occurring at the DNA level are not detected at protein level such as changes in introns and flanking sequences, synonymous codon-changes is not change of electrophoretic mobility can be either genetic or environmental. Also a set of structural genes of organisms responsible for protein to be analysed, which may not be representative of the whole genome.

Vishwanath and Kolte (1997) studied the biomolecule composition among three distinct isolates of *A. brassicae* designated as isolates A, C and D producing distinct spot on mustard. The result revealed that the highly virulent isolate A showed maximum amount of total carbohydrates content and the least virulent D showed least amount of carbohydrates. However isolate C of the fungus, which was moderately virulent showed significantly higher content of total lipids, proteins, RNA and DNA in comparison to isolates A and D. Kumar and Sarma (2004) characterized *Raltonia solanacearum* by using SDS PAGE and resolve the ten isolates in two groups. Dianese, *et al.* (1990) also characterized *R. solanacearum* representing all the biovars based on their membrane protein. Karthikeyan and Thajjudin (2010) used one dimensional SDS PAGE for distinguishing microorganisms including bacteria ecto and ento mychorhiza, etc.

During pathogenesis, pathogen has to overcome the barrier of cell wall and to overcome this barrier, the enzymes capable of degrading cell wall are induced. To understand the mechanism of *Alternaria brassicae* induced changes in the activities of cell wall degrading enzymes viz. polygalacturonase (PGU), cellulase and β1-3 glucanase, was studied by Jain and Dhawan (2008) and finds that role of both PG and cellulase in pathogenesis. Induction of these enzymes during pathogen interaction in the susceptible variety causes cell wall damage. Increase in the activity of β1-3 glucanase in resistant variety may be due to its role in expression of resistance by restricting the growth of pathogen through fungal cell wall hydrolysis/ production of elicitor molecules for inducing various defense mechanism(s) of the host. Garg *et al*., 1999 studied the cell wall degrading enzymes in *Alternaria brassicae* and finds that polygalacturonase and cellulase

decreased in leaf blight resistant cultivar RC-781 and increased in the susceptible cultivar Varuna up to 3 days. In the leaves of both cultivars eleven polypeptides were observed in the absence of *A. brassicae* inoculation. After inoculation in the resistant cultivar RC-781 there was no change in the polypeptide pattern, while in the susceptible cultivar Varuna four polypeptides disappeared (43.7 to 58.5 kDa) only at 3 days after inoculation. Hubballi *et al*., (2011) finds that the activity of cellulolytic enzymes increased with the increase in the age of the culture. Then again, the activity of enzymes produced by the avirulent isolate of pathogens did not decrease and these enzyme activities increased with the increase in the age of the culture.

Cellulase and pectinase production potential of *Aspergillus niger* was studied by Oyeleke *et al.,* (2012) at different pH and temperature to find the optimum pH and temperature for cellulase and pectinase production. The study results showed that highest cellulase activity was obtained on 4th day and least on 7th day. The optimum pH was obtained at pH 4 and lowest cellulase activity was recorded at pH 9. Similarly the optimum temperature was at 50°C with a maximum enzymatic activity and lowest enzymatic activity was obtained at 90°C. In case of pectinase highest enzymatic activity was obtained on the 4th and 5th day and the least on the 6th day. The optimum pH was obtained pH 6 with highest enzymatic activity and the least was recorded at pH 4. Similarly the optimum temperature was at 60°C with a maximum enzymatic activity and lowest enzymatic activity was obtained at 90°C.

Berto *et al.,* (1999) found that *Alternaria brassicae* produced higher quantities (3.2 U/ml) of an inducible extracellular lipase in shaken synthetic medium supplemented with 20 mM methyloleate. After purification, the Molecular weight of the lipase was determined as 80 kDa by SDS-PAGE and estimated at 85 kDa using gel filtration, which suggest that the enzyme may be a monomer. The optimum pH and temperature for activity of the enzyme were 9.0 and 25ºC, respectively. Using umbelliferone esters, the lipase was shown highly specific towards a synthetic substrate with long-chain unsaturated fatty acid. Falony *et al.* (2006) studied extra cellular lipase production in *Aspergillus niger* and finds that the optimum pH and temperature for enzymatic activity were pH 6.0 and 40°C respectively. This enzyme is also stable over a broad pH range, 4 to 7, for a period of 24 hours at 30 °C. The enzyme also exhibited 80 % of its initial activity in neutral and mildly acid media and at temperatures between 20 and 30 °C for a period of 24 hours.

2.13 Molecular Variation

2.13.1 Random Amplified Polymorphic DNA (RAPD)

In general, characterization of pathogen population has been based on morphology, growth characteristics and disease reaction on the host. These methods lack precision, demand more tissue and are not so reliable due to component of interaction with environment. Recent developments in the field of DNA technology provides exciting avenues for identification of host genes and pathogens diversity, phylogenetic relationship within and outside the pathogen population (Bielikova *et al.,* 2002*;* Xu *et al.*, 2003; Zamani *et al.,* 2004 and Gouveia *et al.,* 2005). Among the various molecular techniques, Random Amplified Polymorphic DNA (RAPD) has increasingly been used for the determination of phylogenetic relationships and for the estimation of genetic diversity within fungal population. Random Amplified Polymorphic DNA has been used consistently to determine the genetic variation and subsequently correlating it with the variation in the virulence pattern of the pathogen.

With advancement of molecular techniques, several studies have examined variation in isolates of *Alternaria* species using a range of molecular methods (Pryor and Michailides, 2002; Gherbawy, 2005 and Mercado *et al*., 2006). RAPD analysis allows quick assessment of genetic variability in various taxa and has been used to study inter and intra specific variability among fungal isolates. This analysis proved to be an efficient method for detecting genetic variability of *A. cassiae* and *A. alternata* isolates occurring in *Senna obtusifolia* and also for distinguishing *Alternaria* sp. (Tigano *et al.,* 2003).

RAPD profiling of 55 isolates of *Alternaria* spp. belonging to 13 small spored species and three large spored were carried out using 12 arbitrary primers. The large spored species viz. *A. solani*, *A. porri* and *A. lecunthemi* were differentiated from small spored species by a genetic distance of 0.44 and from each other by the genetic distance of 0.25, indicating that RAPD analysis can be used to analyse the phylogenetic relationship of *Alternaria* sp. (Wang and Zhang, 2003).

The similarity matrix of RAPD analysis of 26 isolates *A. heleanthi* indicated that most of the isolates exhibited 83% similarity coefficient. Isolates were grouped into six groups in the dendrogram generated with the RAPD markers at genetic similarity of 0.34 (Prasad *et al*., 2007). Roberts *et al*. (2000) reveals the RAPD analyses of *A. alternata*, *A. tenuissima*, *A. infectoria* and small spored hostspecific species revealed distinctive RAPD fragment patterns for all species and cluster analysis did resolve these species into distinct clades, which suggests that these taxa constitute well defined species.

Sharma and Tiwari (1998) studied the genetic variation of 20 isolates of *Alternaria brassicae*, *A. brassicicola* and *A. raphani* with RAPD marker. UPGMA analysis of RAPD data showed that isolates collected from geographically distinct regions could be broadly classified into four groups. Intra regional variation between isolates was less apparent. Variation was higher in *A. brassicicola*, as based on RAPD analysis. RAPD analyses of *Alternaria* by Cooke *et al*. (1998) and Weir *et al*. (1998) evaluated the genetic diversity of several species but did not assess the congruence of morphological and genetic characters.

The RAPD questioned to its lack of total reproducibility (Lamboy, 1994), but it can been utilized to generate unique PCR products or amplicons in any living organism especially filamentous fungal species or strains of interest to be converted into species- or strain-specific sequence-characterized amplified region (SCAR) markers (Abbasi *et al.,* 1999; Lecomte *et al*., 2000; Li *et al*., 1999 and Schilling *et al.*, 1996). SCAR markers differ from RAPD markers in that SCAR primers are designed based on known DNA sequences of the organism of study. This allows for the development of sensitive and diagnostic assays to amplify specific fungal DNA in laboratory cultures as well as field samples containing mixed DNA because primers anneal specifically to fungal sequences.

Jimenez-Gasco and Jimenez-Diaz (2003) developed specific primers and polymerase chain reaction (PCR) to identify *F. oxysporum* f. sp. *ciceris* and pathogenic races 0, 1A, 5, and 6. Sequence characterized amplified region (SCAR) primers for specific PCR were developed by cloning and sequencing of *F. oxysporum* f. sp. *ciceris* and race-specific random amplified polymorphic DNA (RAPD) bands. Each cloned RAPD marker was characterized by southern hybridization analysis of EcoRI-digested genomic DNA of a subset of *F. oxysporum* f. sp. *ciceris* and nonpathogenic *F. oxysporum* isolates. All except two cloned RAPD markers consisted of DNA sequences that were found highly repetitive in the genome of all *F. oxysporum* f. sp. *ciceris* races and *F. oxysporum* f. sp. *ciceris* isolates. The specific primer pairs amplifies a single 1,503bp product from all *F. oxysporum* f. sp. *ciceris* isolates; and single 900bp and 1,000bp products were selectively amplified from race 0 and race 6 isolates, respectively. The specificity of these amplifications was confirmed by hybridization analysis of the PCR products. A race 5-specific identification assay was developed using a touchdown-PCR procedure. A joint use of race 0 and race 6 specific SCAR primers in a single-PCR reaction together with a PCR assay using the race 6 specific primer pair correctly identified race 1A isolates for which no RAPD

marker had been found previously. The specific SCAR primers and PCR assays developed were clearly identified and differentiated the isolates of *F. oxysporum* f. sp. *ciceris* and each of its pathogenic races 0, 1A, 5, and 6.

Devi *et al.,* 2013 developed the SCAR marker for the detection of *Aspergillus flavus*. In this studies an amplified RAPD products of 600 bp were obtained in *A. flavus* isolates using a random primer OPB-11 was cloned in pGEMT easy vector and sequenced. Based on sequences, six primers were designed, out of which a primer pair Asp f1 and Asp r2 amplified a sequence of 490 bp which was specific to *A. flavus*. This sequence characterized amplified region (SCAR) marker was sensitive and could detect small quantities of *A. flavus* DNA as low as 10 to 25 ng with high efficiency. These markers were also clearly distinguishing *A. flavus* from other fungal plant pathogens, including different *Aspergillus* spp. This SCAR marker was recommended for the diagnosis of *Aspergillus flavus* in post harvest samples for the detection of aflatoxin.

DNA primers specific for *Verticillium dahliae* were designed by using the DNA sequence from a RAPD fragment. The amplification results indicated that this amplicon is shared by all tested *V. dahliae* isolates but not by the closely related *Verticillium* species like *V. albo-atrum*, *V. tricorpus* and *V. nigrescens*, nor by any other plant wilt pathogens or fungi such as *Fusarium oxysporum*, *F. moniliforme* and *Sclerotinia sclerotiorum.* The limit of detection of this PCR based assay was between 50 to 500 copies of target sequence in the form of purified DNA, or 450 conidia. As few as 17 microsclerotia in 1 g of silica sand was detectable here, while a detection limit of 1±2 microsclerotia per gram of soil has been reported, using conventional methods (Li *et al.*, 1999).

2.1. 11 Internal Transcribed Spacer (ITS) region:

Analysis of ribosomal DNA (rDNA) sequences has become a common tool in modern systematics and has been used to establish molecular phylogenetic relationships within many groups of fungi (Miyamota and Cracraft, 1991; Moritz and Hillis, 1990 and White *et al*., 1990). For example, analysis of the relatively conserved nuclear 18S rDNA sequence has been used to describe family and genus relationships within the *Pleosporales*, and revealed a close phylogenetic relationship between *Pleospora* (anamorph *Stemphylium*) and *Alternaria* (Berbee, 1996; Morales *et al*., 1995). For analysis of fungal taxa at or below the species level, the more variable internal transcribed spacer (ITS) region is commonly used. This region has been used to examine phylogenetic relationships among *Alternaria* species that produce host-specific toxins

(Kusaba and Tsuge, 1995) or that is pathogenic on crucifers (Jasalavich *et al*., 1995), as well as among other species of the *Pleosporales* (Khashnobish and Shearer, 1996 and Morales *et al*., 1995).

Nucleotide sequences of the ribosomal DNA (rDNA) internal transcribed spacers (ITS) 1 and 2 and a 1068 bp section of the beta tubulin gene divided seven designated species of *Alternaria* into five taxa. *Stemphylium botryosum* formed a sixth closely related taxon. Isolates of *A. linicola* possessed an identical ITS sequence to one group of *A. solani* isolates and two clusters of *A. linicola* isolates, revealed from beta tubulin gene data to show minor variation, were as genetically similar to isolates of *A. solani* as they were to each other. We suggest, therefore, that *A. linicola* falls within the species *A. solani*. Similar results suggest that *A. linicola* falls within the species *A. alternata*. RAPD analysis of the total genomic DNA from the *Alternaria* spp. concurred with the nucleotide sequence analyses. An oligonucleotide primer (ALP) was selected from the rDNA ITS1 region of *A. linicola* / *A. solani*. PCR with primers ALP and ITS4 (from a conserved region of the rDNA) amplified 536 bp fragment from isolates of *A. linicola* and *A. solani* but not from other *Alternaria* sp. nor from other fungi which may be associated with linseed. These primers amplified an identical fragment, confirmed by Southern hybridization, from DNA released from infected linseed seed and leaf tissues. These primers have the potential to be used also for the detection of *A. solani* in host tissues (McKey *et al*., 1999).

Morales *et al.* (1992) have shown that the highly virulent and weakly virulent strains of *Leptosphaeria maculans* may actually be different species. It is based on the ITS1 sequences alone. The phylogenetic relationship among several *Leptosphaeria* species has been studied based on the 5.8S rDNA and ITS regions as well as the 18S rDNA (Morales *et al.,* 1995).

The sequences coding for the nuclear 18S rRNA, 5.8S rRNA, and the internal transcribed spacers (ITS1 and ITS2) were amplified by the polymerase chain reaction and sequenced for one isolate each of *Alternaria brassicae, A. brassicicola, A. raphani, A. alternata* and *Pleospora herbarum.* The 5.8S rDNA sequences from the four *Alternaria* species were identical and differed at only one base pair from that of *P. herbarum.* The internal transcribed spacer sequences, especially ITS1, were very variable in both base composition and length. The 18S rDNA sequences were highly conserved, but enough variability was present to distinguish genera clearly. Phylogenetic analysis of the sequence data sets by both parsimony and maximum likelihood methods clearly separated genera and species. All of the *Alternaria* species were

closely related. *Pleospora* also appeared to be more closely related to *Alternaria* than to *Leptosphaeria* (Jasalavich *et al*., 1995).

The ITS sequences within the ribosomal DNA (rDNA) region were targeted to delineate genetic variability among eight *Alternaria* species that cause economically important diseases in crops. The rDNA regions of Alternaria species comprising of rRNA genes and the ITS regions were cloned and sequenced. Phylogenetic relationship based on the rDNA sequences and PCR-RFLP of amplified rDNA sequences clustered eight species of Alternaria into three major groups.

3. MATERIALS AND METHODS

In the present study the collection of *Alternaria brassicae* disease specimen were carried out in different locations of India. Isolation and purification of pathogen, multiplication and maintenance of purified isolates, morphological, pathogenic cross infectivity, genetic diversity of pathogen by the molecular marker such as Random amplified polymorphic DNA (RAPD) and sequencing of ITS region was undertaken in the Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi. Field evaluation of disease severity on different mustard, cabbage and cauliflower varieties were included in the present study. The materials and methodologies adopted for the experimentations are described in this chapter. Composition and preparation of the media and buffer are given in the Appendix.

3.1 Location of Culture collection

Disease infected leaf samples of mustard, cabbage and cauliflower were collected from different locations of India. The details of the locations and hosts were given below in Table 3.1

3.2 Materials:

3.2.1 Glassware

The glasswares used in the present study like Petri plates (90 mm), conical flasks, test tubes, beakers, funnels and pipettes etc. are Borosil and Corning brands. The glasswares were cleaned thoroughly with detergent solution and washed in running tap water, rinsed with distilled water and dried in hot air oven.

3.2.2 Equipments

Equipments used during the course of investigation included compound microscope, pestle and mortar, grinder, refrigerator, autoclave, hot air oven, B.O.D., incubator, laminar air flow, weighing balance, LPG gas burner, spectrophotometer, haemocytometer, shaker, water bath, thermocycler, gel-electrophoresis unit, hot plate etc. Small instruments like inoculation needle, scalpel, cork borer, razor, and glass cavity slides, glass rods, syringe, etc.

Table 3.1 List of *Alternaria brassicae* isolates with their host and their location of collection used in this study

Sl. No.	Isolates Code	Host	Location	State
1	AB-1	Cauliflower	Ghaziabad	Uttar Pradesh
2	AB-2	Cauliflower	Alipur	Uttar Pradesh
3	AB-3	Cauliflower	Noida	Uttar Pradesh
4	AB-4	Cauliflower	Meerut	Uttar Pradesh
5	AB-5	Cauliflower	Bagpat	Uttar Pradesh
6	AB-6	Cauliflower	Saharpur	Uttar Pradesh
7	AB-7	Cauliflower	Kanpur	Uttar Pradesh
8	AB-8	Cauliflower	Lucknow	Uttar Pradesh
9	AB-9	Mustard	Rampur	Uttar Pradesh
10	AB-10	Mustard	IARI	Delhi
11	AB-11	Cabbage	IARI	Delhi
12	AB-12	Cauliflower	IARI	Delhi
13	AB-13	Cauliflower	Najabgarh	Delhi
14	AB-14	Cauliflower	Saraikalakhan	Delhi
15	AB-15	Cauliflower	Mother dairy field	Delhi
16	AB-16	Cauliflower	Jaipur	Rajasthan
17	AB-17	Mustard	Bharatpur	Rajasthan
18	AB-18	Cauliflower	Kalyani	West Bengal
19	AB-19	Cauliflower	Hoogli	West Bengal
20	AB-20	Cauliflower	Kolkata	West Bengal
21	AB-21	Cauliflower	Sonepet	Haryana
22	AB-22	Mustard	Mewat	Haryana
23	AB-23	Mustard	Hisar	Haryana
24	AB-24	Cabbage	Gurgaon	Haryana
25	AB-25	Mustard	Karnal	Haryana
26	AB-26	Mustard	Pant Nagar	Uttarakhand
27	AB-27	Cabbage	Pant Nagar	Uttarakhand
28	AB-28	Cauliflower	Pant Nagar	Uttarakhand
29	AB-29	Cabbage	Nainital	Uttarakhand
30	AB-30	Cauliflower	Nainital	Uttarakhand
31	AB-31	Mustard	Kashipur	Uttarakhand
32	AB-32	Cauliflower	Almora	Uttarakhand
33	AB-33	Cabbage	Hyderabad	Andhra Pradesh
34	AB-34	Cauliflower	Nagpur	Maharashtra
35	AB-35	Cabbage	Dindigul	Tamil Nadu
36	AB-36	Cabbage	Palani	Tamil Nadu
37	AB-37	Cauliflower	Coimbatore	Tamil Nadu
38	AB-38	Cabbage	Theni	Tamil Nadu
39	AB-39	Cabbage	Hosur	Tamil Nadu
40	AB-40	Cauliflower	Palakad	Kerala

3.2.3 Sterilization

All types of glassware including Petri plates, test tubes, conical flasks and pipettes used in the present investigation were sterilized in a hot air oven at 160°C for 2 h. Inoculation needle was flame sterilized. Nutrient media was sterilized at 1.1 kg/cm^2 (121.6° C) for 15 minutes in an autoclave.

3.3 Isolation and purification of *A. brassicae* isolates.

The pathogen *Alternaria brassicae* was isolated from diseased leaves as per methods described by Dhingra and Sinclair (1985). Diseased leaves of mustard, cabbage and cauliflower were collected from different agro-climatic regions of India. Diseased leaves were cut into small bits of 2-3mm long. The leaf bits washed thoroughly in sterilized water to remove the dirt. Then the bits were surface sterilized with 0.1% mercuric chloride solution for 20-30 seconds and subsequently washed 2-3 times in sterile distilled water to remove the traces of mercuric chloride. Excess moisture was removed by placing this infected leaf bits in between two pre-sterilized blotting paper in laminar flow. Surface sterilized pieces (2-3) were placed in sterilized petridishes containing previously sterilized potato dextrose agar (PDA) medium. Inoculated petridishes were incubated at 29°C in BOD incubator. As soon as the fungal growth was visible around the inoculation pieces, it was transferred to another petridishes with sterilized PDA medium. After 2-3 days, they were again transferred to fresh PDA slant and incubated at 29°C. The cultures were purified by single spore method (Toussoun and Nelson, 1976), and they were maintained by PDA slants. These slants were periodically transferred at one month interval to new slants. Cultures were maintained on sterile potato dextrose agar slants in refrigerator at 4°C. The isolates of the fungus were identified on the basis of mycelial and conidial characters.

Forty isolates of *Alternaria brassicae* were taken for this study. Out of nineteen were taken from lab no 26, from where previous collections were made, the remaining isolates were collected from different parts of India as above mentioned location.

3.4 Cultural variability

The cultural characteristics of the fungus was studied in three different media viz., Potato Dextrose Agar (PDA), Carrot Potato Agar (CPA) and Cauliflower Leaf Extract Agar (CLEA) media following the methodology given by Alcorn (1983a and 1983b), Sivanesan (1987) and Karimi (2003) with certain modifications like cultural characteristics *viz,* colony colour, texture and radial growth were recorded on seven day after inoculation.

3.4.1 Colony colour

The observations on colony colour were recorded seven days after inoculation. The colour of the colony was observed from the top and bottom side of the culture plates. Based on the colony colour, the cultures were assigned to different groups.

3.4.2 Colony texture

Observations for the colony texture were recorded on seventh day after inoculation when the growth covered full petriplate. The isolates were designated to different groups based on nature of the texture of their mycelial growth and appearance.

3.4.3 Radial growth

The average growth was recorded in terms of diameter of Petriplate. For radial growth measurement, 20 ml of sterilized medium was poured in sterilized petriplates. After solidification, the petriplates were inoculated with seven day old culture block of 2 mm diameter placed inverted in the centre of the plate and incubated at 29±1°C in BOD incubator with alternate light and dark for 12 hrs daily. Radial growth pattern of the *A. brassicae* colonies were observed on seven day after inoculation. The average growth was recorded in terms of diameter of petriplate. Experiments were conducted in three replications. Based on radial growth, the cultures were assigned to different groups.

3.4.4 Morphological variability

Morphological variability of *A. brassicae* was evaluated by studying various characteristics such as size of conidia, beak and number of septa. The sporulation pattern of the

isolates was also studied. Media plates inoculated with pathogen isolates were incubated for fifteen days at 28°C under continuous fluorescent light. The number of conidia were counted using haemocytometer. Conidia produced per unit surface area were estimated using the formula given below.

$$\text{Conidia produced per unit surface area } (mm^2) = \frac{\text{Number of conidia } ml^{-1} \text{ suspension} \times \text{Total surface area from which conidial suspension was derived}}{\text{Volume of water to make suspension}}$$

3.4.5 Calibration of the microscope and measurements

Calibration of microscope (Leitz-monocular microscope) was done with the help of ocular micrometer (1cm = 100 div.) and stage micrometer (1 div. = 0.01 mm). Divisions of the ocular micrometer were super imposed or synchronized with small divisions of stage micrometer and the numbers of ocular divisions coinciding with the divisions of the stage micrometer were counted. Fifty readings were taken and average of these measurements were taken as normal for calculation of the R.F. value of the microscope. Calibration was done with 10X, 40X and 100X (oil immersion) objective and with 10X eye piece lens. The slide views in the present study were from compound microscope.

3.4.6 Measurements

Measurements of the morphological features were made with the help of ocular micrometer on a calibrated microscope. Twenty five measurements were taken in each case to calculate the average minimum and maximum size of the spores.

3.5 Pathogenic cross infectivity study

Pathogenic variation in the isolates of *A. brassicae* was ascertained on the basis of the ability of each of the isolates to cause diseases in fifteen different varieties of mustard, cabbage and cauliflower. The study was performed by detached leaf technique described by Sharma *et al.*, 2004. In this method third or fourth detached leaves from plants of mustard, cabbage and cauliflower grown in IARI field were used. Here area of approximately 3 mm^2 on the upper leaf

surface was gently scratched and 1 ml of spore suspension (5 x 10^3 spores/ml) was injected by using sterilized syringe. The spore suspension was applied at six places on each leaf. The treated leaves were kept in polyethylene bags having moist blotting papers to maintain relative humidity (85-100%) at 27° C. The symptoms started appearing after fifth day of inoculation, symptoms rated visually by using 1-9 scale (1= no infection, 9=89-100% infection), plants rated 1-3 are resistant, 4-6 are moderately resistant and 7-9 as susceptible. Entries with > 75% susceptible plants are classified as susceptible, 50-74.9% as moderately susceptible, 25-49.9% as moderately resistance, 1-25% resistant and 0-0.99% as highly resistant.

3.5.1 Disease scoring

The disease scoring was evaluated by using 1-9 grade scale. The scale consists of nine broad categories designated by numerals 1 to 9 (Table 3.2). Based on the rating the percent disease index was calculated by using the below formula.

$$\text{Per cent Disease Index (PDI)} = \frac{\text{Sum of individual ratings}}{\text{Total number of plants/ leaves observed}} \times \frac{100}{\text{Maximum disease grade}}$$

3.6 Biochemical variations

Identification of variations in *Alternaria brassicae* was done using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

3.6.1 Stock solutions for SDS-PAGE

(a) Extraction buffer (pH 7.5)

Tris-HCl	:	50 mM
EDTA (Na_2)	:	50mM
BME	:	0.3%
PMSF	:	2mM
PVP	:	0.5%

Table 3.2 Disease rating scale (1-9) rating scale for black leaf spot of crucifers

Rating/ Grade	Lesion Size	Disease reaction
1	No infection / symptom	Resistant
2	Few spots, 1 to 5 % infection	Resistant
3	Few spots, less than 10% leaf area	Resistant
4	Spots with pale outer zone, up to 20% area	Moderately resistant
5	Spots with pale outer zone, up to 30% area	Moderately resistant
6	Lager spot with yellow hole, up to 50 % infection	Moderately resistant
7	Drying up of the leaves, up to 75% infection	Susceptible
8	Collision of spots and drying up of the leaves, up to 90% infection	Susceptible
9	Collision of spots and complete drying up of the leaves, collision of spots and 100% infection	Susceptible

The media were autoclaved at 120^0C in 15 p.s.i. for 20 minutes.

(b) 30% Acrylamide and bis-acrylamide stock solution:

29.2g of acrylamide and 0.8 of N, N'- methylene bis acrylamide were dissolved in 100ml of distilled water. These were stirred in magnetic stirrer and warmed slightly so as to dissolve completely. The solution was filtered and stored at 4^0C in a dark coloured bottle.

(c) 10% SDS:

10g of Sodium dodecyl sulphate was dissolved in 100ml of distilled water.

(d) Stacking gel buffer: 0.5M Tris-HCl (pH 6.8)

(e) Separating gel buffer: 1.5M Tris-HCl (pH 8.8)

(f) Electrophoresis: (pH 8.3) Tris-Glycine buffer)

Tris-HCl : 25mM

Glycine	:	192mM
SDS	:	0.1%

(g) Sample buffer (2X)

Tris-HCl (pH 6.8)	:	62mM
SDS	:	2%
Glycerol	:	10%
BME	:	5%
BPB	:	0.1%

(h) Staining solution

50% Methanol

10% Glacial acetic acid

0.1% CBB-R250

(i) Destaining solution

10% methanol

7% Glacial acetic acid

(j) 10% APS

Ammonium per sulphate (0.1 g) was dissolved in 1 ml water.

3.6.2 Protein extraction from the fungal mat

The mycelial mats were ground in pre-chilled sterilized pestle and mortar into fine powder with liquid nitrogen and transferred to centrifuge tubes, containing extraction buffer (Tris-HCl 0.05 M pH 7.4, glycerol 5%, sodium dodecyl sulphate (SDS) 0.5% and mercaptoehanol 0.1%). The tubes were allowed to stand for one hour on ice. The samples were then centrifuged at 10,000 rpm for 45 min, at 4°C the clear supernatant was collected. To these seven volumes cold acetone was added and kept at -20°C for overnight. The proteins were precipitated by centrifugation at 10000 rpm for 20 min at 4°C, the pellet was washed two times

with cold acetone and air dried. The pellet was dissolved in sample buffer for SDS-PAGE and samples were loaded after denaturation. The gel was stained in coomassie blue solution.

3.6.3 Preparation of SDS Polyacrylamide Gel

Polyacrylamide gel was prepared from the stock solutions by mixing them in following proportion. Gel was poured immediately after addition of TEMED and APS for polymerization.

3.6.3.1Composition of 10% separating gel (20ml)

30% Acrylamide stock	:	6.6ml
Separating gel buffer	:	5.0ml
10% SDS	:	100µl
10%APS	:	200µl
TEMED	:	10µl
Distilled water	:	8.5ml

3.6.3.2 Composition of 5% stacking gel

30% Acrylamide stock	:	1.3ml
Stacking gel buffer	:	2.0ml
10% SDS	:	80.0µl
10%APS	:	80.0µl
TEMED	:	10.0µl
Distilled water	:	4.53ml

3.6.3.3 Sample preparation

About 50 µl of crude protein sample was mixed with 2X sample buffer in 1:1 ratio. Sample was boiled in water bath for 5min. and centrifuged at 10,000 rpm for 10 min and loaded on gel. Molecular weight of marker in the range of 14-100 kDa (Bangalore, Genei) was 10µl/well) used as standard.

3.6.3.4 Electrophoresis

Gel was pre-run for 15min at 80V. Samples were loaded and electrophoresed at 80 Volts till the sample is in stacking gel and voltage raised up to 120 volts for separating gel. Allow the gel to run till the dye reach 0.5cm (about 7 – 8 hrs.) from the lower edge of the gel.

3.6.3.5 Staining and destaining of SDS-poly-acrylamide gel.

After the electrophoresis, the gel was stained in staining solution containing Coomassive brilliant blue (R250) for overnight. The gel was destained in destaining solution (3%Nacl).

3.6.3.6 Scoring and data analysis

The SDS-PAGE Gels were scored on the basis of the presence (1) or absence (0) of each band for all isolates. All gels were repeated at least twice and only reproducible bands were considered for analysis. The similarity coefficient obtained were transformed into Olsen-Jaccard's pattern i.e. those strains having the same banding pattern were given 1 and those not having that band were designated 0 and this 0-1 pattern was utilized to obtain the Euclidian distance matrix among the strains. UPGMA dendrogram was prepared based on the similarity co-efficient value with the help of NTSYS pc version 2.02i software.

3.6.4 Enzyme assay

For the assay of enzyme activity the isolates *Alternaria brassicae* were grown on minimal synthetic medium (MSM). The minimal synthetic broth (MSB) contained the following components (in grams per liter): MgSO4. $7H_2O$- 0.2; K_2HPO_4-0.9; KCl- 0.2; NH_4NO_3 -1.0; $FeSO_47H_2O$-0.002; $MnSO_4$-0.002 and $ZnSO_4$-0.002. The medium was supplemented with the appropriate carbon source (0.5 %) and the pH was set to 6.3 (50 mM phosphate buffer). with respective carbon source (Cellulose for cellulase, pectin for pectin and olive oil for lipase). The cultures were harvested after 4day of inoculation. The culture filtrate was filtered through Whatman No.44 filter paper and centrifuged at 12000 rpm for 10 min to get cell free culture filtrate. These culture filtrates were used for assay of enzymatic activities.

3.6.4.1 Cellulase Assay

The qualitative assay was performed before the quantitative assay, the qualitative assay performed in the petri plates. Minimal synthetic media prepared as per the above composition. pH of the media was set at 6.5 and autoclaved at 15 lb/in^2 for 15 min. the autoclaved media was poured into petri plates. After media solidification *Alternaria brassicae* was inoculated and incubated at 29°C in an inverted position for 2-5 days. After the visible growth seen the plates were flooded with 1% aqueous solution at hexadecyltrimethyl ammonium bromide/congo red. The plates were kept in shaker for 4 hr. Then the aqueous solution were drained out and poured with 1% NaCl and incubated for another 1 hr and drained out. Clear areas around the colony of *A. brassicae* indicate production of respective enzymes.

Cellulose activity was assayed by measuring the release of reducing sugar with DNS (Miller, 1959). The assay mixture contained 1 ml of 0.5% cellulose (Sigma Co.) suspended in 50Mm citrate phosphate buffer (pH 4.8) and 1 ml of culture filtrates of different *Alternaria Brassicae* strains in 15 ml test tubes. The reaction mixture was incubated for 30 min at 50^0 C and then centrifuged at 12000rpm for 15 min at 4^0 C. The reaction was arrested by adding 3 ml of 1% DNS (dinitrosalicylate) reagent in 1 m NaOH and followed by heating for 10 min at 100^0 C to develop the red-brown colour. While it was hot 1ml of 40% Rochelle salt (potassium sodium tartrate) was added to stabilise the color. The blanks were made in the same way using distilled water bath, the absorbance was measured with a spectrophotometer (Systronics spectrophotometer) at 540 nm and the glucose content was obtained by comparing with glucose stanadard prepared by the same procedure. One unit of cellulose activity was defined as the amount of enzyme in one 1 ml of the reaction mixture that released 1µmol of reducing sugar under the assay condition.

3.6.4.2 Lipase Assay

Qualitative assay performed in the petri plates. For lipase assay the isolates *Alternaria brassicae* were grown on minimal synthetic medium (MSM). The minimal synthetic broth (MSB) contained the following components (in grams per liter): MgSO4. 7H_2O- 0.2; K_2HPO_4- 0.9; KCl- 0.2; NH_4NO_3 -1.0; $FeSO_4.7H_2O$-0.002; $MnSO_4$-0.002 and $ZnSO_4$-0.002 and olive oil as carbon source. Minimal synthetic media prepared as per the above composition. pH of the media was set at 6.5 and autoclaved at 15 lb/in^2 for 15 min. the autoclaved media was poured

into petriplates. After media solidification *Alternaria brassicae* was inoculated and incubated at 29°C in an inverted position for 2-5 days. After the visible growth seen the plates were flooded with 1% aqueous solution at hexadecyltrimethyl ammonium bromide/congo red. The plates were kept in shaker for 4 hr. Then the aqueous solution were drained out and poured with 1% NaCl and incubated for another 1 hr and drained out. Clear areas around the colony of *A. brassicae* indicate production of respective enzymes.

For quantitative determination, titrimetric method was used (Sugihara *et al*., 1991). Here olive oil used as a substrate (olive oil emulsified 3% gum Arabic). 75 ml of 2% polyvinyl alcohol was homogenized with 25 ml of olive oil and used as a substrate. The substrate emulsion of 5 ml and 4 ml of 0.1 M of phosphate buffer at pH 7.0 was incubated at 37°C for 20 minutes. The reaction was terminated by the addition of acetone- alcohol (1:1) and titrated against 0.05N NaOH. The boiled enzyme solution in the reaction mixture was used as control. 1 unit of lipase activity was defined as the amount of enzyme released from fatty acid in one minute under standard assay condition.

3.6.4.3 Pectinase Assay

Qualitative assay performed in the petri plates. It was formulated the pectinase screening agar medium (PSAM): 1gm pectin, 0.3 gm Diammonium orthophosphate;0.2 gm; KH_2PO_4 ; 0.3 gm K_2HPO_4, 0.01 gm $MgSO_4$ and 2.5 gm agar(for 100 ml) The initial pH of medium was adjusted to 4.5. This medium was sterilized and distributed aseptically in Petri dishes. The Petri dishes containing PSAM were inoculated and incubated at 37°C for 2-3 days. At the end of the incubation period, plates were stain with 50 mM iodine for result.

Pectinase activity was assayed by the colorimetric method of Miller. Briefly, 0.5ml of cell free supernatant was incubated with 0.5ml of pectin in 0.1M acetate buffer with pH 6.0 and the reaction mixture was incubated at 40°C for 10 minutes in static condition. After adding 1ml of DNS reagent, the mixture was boiled for 5 min at 90°C. The reaction was stopped by adding 1ml of Rochelle's salt. Then the mixture was diluted by adding 2ml of de-ionized water. The absorbance was measured Spectrophotometrically at 595 nm. A standard graph was generated using standard glucose solution. One unit of Pectinase activity was defined as the amount of enzyme which liberated 1µm glucose per min.

3.6.4.4 Brassinin assay

The anti-fungal activity of brassinin to and *A. brassicae* (isolate AB-16) was investigated using the following two assays.

3.6.4.4.1 Mycelial radial growth assay

A dimethylsulfoxide (DMSO) solution of brassinin (final concentration 0.12–0.50 mM) was added to V8 agar medium and poured petri plates. Fungal culture (*A. brassicae*) disc was placed on the petri plates contain medium. The plates were incubated under constant light, at 25±2°C. Control plates containing only DMSO were prepared and incubated similarly. The diameter of mycelial growth (in mm) was measured after 72 h at 24-h intervals up to 168 h.

3.6.4.4.2 Spore germination inhibition assay

DMSO (control) or a DMSO solution of brassinin (final concentration 0.12–0.50 mM) was added to spores suspensions of *A. brassicae* isolates ($1.8X10^5$ spores ml_1) in minimal medium containing 1% Tween 80 and incubated on a shaker at 25±2°C. The germinated and ungerminated spores were counted in ten random fields of each well with an inverted microscope at 40X magnification.

3.6.4.5 Statistical analysis:

The data will be analyzed for significance using student's t-test. Results will be expressed as mean of three replications. CRD and Regression matrix will be used as statistical tools for interpretation of data.

3.6.5 Fungal protein quantification by Bradford assay

Four spectroscopic methods were routinely used to determine the concentration of protein in a solution. These include measurement of the protein's intrinsic UV absorbance and three methods which generate a protein-dependent color change; the Lowry assay, the Smith copper/bicinchoninic assay and the Bradford dye assay. Among the four methods Bradford assay is faster, involves fewer mixing steps, does not require heating, and gives a more stable colorimetric response than the assays described above. This assay is based on the use of a dye,

Coomassie Brilliant Blue G-250, to which protein binds, altering the light absorbance properties of the dye. When the dye is prepared as an acidic solution (in 85% phosphoric acid), it maximally absorbs light with a wavelength of 465 nm. Addition of protein results in a shift of the dye's absorption maximum to 595 nm. As the protein concentration increases, the absorbance of light at 595 nm increases linearly. This increase in absorbance can be measured in a spectrophotometer. Although the absorbance of Coomassie blue dye at 595 nm is proportional to the amount of protein bound, it is necessary to establish a correspondence between absorbance values and known amounts of protein. To do this, you will prepare a series of *protein standards* – dilutions of a protein solution of known concentration.

In order to measure and plot a standard curve of protein concentration versus absorbance at 595 nm, a series of dilutions of the BSA protein standard stock solution was prepared. The easiest way to solve for the volume of protein stock solution required for each dilution is to use the formula $C_1V_1 = C_2V_2$. C_1 is the concentration of the protein stock solution, V_1 is the volume of the stock solution required, C_2 is the concentration of the diluted sample, and V_2 is the volume of the diluted sample. The concentration of the stock solution (C_1) is 100 µg/ml, the concentration of the diluted sample is (C_2), and the volume of the diluted sample is fixed at 200 µl. Therefore, solving for the volume of stock solution required: $V_1 = C_2V_2/C_1$

Protein Standards - Protein standards were prepared in the same buffer as the samples to be assayed. A convenient standard curve was made using bovine serum albumin with concentrations of 0, 10, 20, 30, 40, 50 µg/ml for the microassay (extinction coefficient of BSA is 0.667).

3.6.5.1 Statistical analysis

The data obtained from spectrophotometer were used for the determination of protein concentration in fungal sample.

3.7 Molecular characterization

3.7.1 Fungal multiplication

Potato Dextrose Broth (PDB) was used as medium for mycelial growth of fungus for which DNA was extracted. 150 ml of medium was dispensed in 250ml conical flasks and sterilized at 121.6°C at 15 lb for 20 min. Each flask was inoculated with 10 mm mycelial disc of the fungus taken from the actively growing cultures of different isolates on PDB. The inoculated flasks were incubated for seven days at 27 ± 1°C in shaker incubator (Kuhner ISF-I-V, Switzerland). After incubation, the mycelial mats were harvested by filtering through sterilized Whatman paper No.1.

3.7.2 DNA extraction

DNA extraction was done by Cetrimide Tetradecyl Trimethyl Ammonium Bromide (CTAB) method given by Murray and Thompson (1980). One gram mycelial mat was weighed and ground to fine powder form in liquid nitrogen by using pre-chilled pestle and mortar. The powdered mycelium was transferred into sterilized centrifuge tubes conferring 10 ml of pre heated (65°C), 2 per cent CTAB extraction buffer to make a slurry. These tubes were incubated at 65°C for one hr, after incubation, 10 ml of Phenol: chloroform: isoamyl alcohol (25: 24: 1 v/v) was added to the slurry and gently mixed. The tubes were then centrifuged at 13,000 rpm for 15 minutes at 4 ºC. Upper aqueous phase containing nucleic acid was precipitated with 0.8 volumes of isopropanol and centrifuged at 13,000 rpm for 15 minutes at 4 ºC. The pellet obtained was washed with 70 per cent ethanol and air dried. Pellet was then dissolved in TE buffer and stored at -20°C.

3.7.3 Purification of DNA

Ribonucleic Acid (RNA) in the total nucleic acid extracted was removed by RNase treatment. The reaction mixture was as follows: Total nucleic acid ~ 200 µl; RNase- 2 µl, the reaction mixture was incubated at 37°C for one hr. Equal volume of phenol: chloroform: isoamylalchol (25:24:1) was added to the DNA mixture and centrifuge at 10,000 rpm for 5min at 4°C. Aqueous phase was collected in fresh tube and 3M sodium acetate followed by chilled ethanol 95% was added. DNA was pelleted and washed with 70% ethanol and dissolved in 200µl TE buffer. DNA concentration and purity of samples was determined by measuring ultraviolet absorbance at 260 nm and 280 nm in a spectrophotometer (Kontron instruments, Italy) and also by visual observation on Ethidium bromide.

3.7.4 Random Amplified Polymorphic DNA analysis

3.7.4.1 Standardization of RAPD protocol

Random Amplified Polymorphic DNA (RAPD) analysis conditions for *Alternaria brassicae* isolates were standardized. Protocol for PCR was optimized by varying different parameters.

3.7.4.2 Optimization of Polymerase Chain Reaction (PCR) reaction

Composition of PCR reaction was optimized by varying the concentration of template DNA (25 ng, 50 ng and 75 ng) along with three concentrations of $MgCl_2$ (100 µM, 50 µM, 25 µM), dNTPs (25 µM, 50 µM, 100 µM) and primer (5 µM, 10 µM and 15 µM) were used with 1 unit *Taq* DNA polymerase and 10X reaction buffer (Fermentas Life Sciences, Canada). Different PCR profiles were tested for obtaining best amplification of nucleic acid of the *A. brassicae* isolates under investigation. The polymerase chain reaction was performed using Biorad Gene Cycler™. The amplification was performed with the following conditions: Template DNA of 50 ng, 25 mM $MgCl_2$, 10 mM dNTP, 0.5 µl of primer (10 nucleotide length), 2.5 units of *Taq* DNA polymerase, 10X PCR buffer in reaction volume of 15 µl. The standardized temperature profile of 94°C for 5 minutes followed by 32 cycles of 93°C for 45 sec 34°C for 30 sec. and 72°C for 50 sec with a final extension of 72°C for 8 minutes.

3.7.4.3 Primer screening and selection

Preliminary primer screening was carried out and 40 primers from OPA, OPC and OPE series (Operon Technologies, Inc., USA) were employed for molecular variation analysis (Table 3.3). The primers that gave reproducible and scorable amplifications were used in the analysis of genetic variability of the isolates.

Table 3.3 List of 10 mer aribitary RAPD primer used for screening of *A. brassicae* isolates

Sl .No.	Name of the of Primers	Primer Sequences (5’-3’)
1	OPA-01	CAGGCCCTTC
2	OPA-02	TGCCGAGCTG
3	OPA-03	AGTCAGCCAC
4	OPA-04	AATCGGGCTG
5	OPA-05	AGGGGTCTTG
6	OPA-06	GGTCCCTGAC
7	OPA-07	GAAACGGGTG
8	OPA-08	GTGACGTAGG
9	OPA-09	GGGTAACGCC
10	OPA-10	GTGATCGCAG
11	OPA-11	CAATCGCCGT
12	OPA-12	TCGGCGATAG
13	OPA-13	CAGCACCCAC
14	OPA-14	TCTGTGCTGG
15	OPA-15	TTCCGAACCC
16	OPA-16	AGCCAGCGAA
17	OPA-17	GACCGCTTGT
18	OPA-18	AGGTGACCGT
19	OPA-19	CAAACGTCGG
20	OPA-20	GTTGCGATCC
21	OPC-01	TTCGAGCCAG
22	OPC-02	GTGAGGCGTC
23	OPC-03	GGGGGTCTTT
24	OPC-04	CCGCATCTAC
25	OPC-05	GATGACCGCC
26	OPC-06	GAACGGACTC
27	OPC-07	GTCCCGACGA
28	OPC-08	TGGACCGGTG
29	OPC-09	CTCACCGTCC
30	OPC-10	TGTCTGGGTG
31	OPE-01	CCCAAGGTCC
32	OPE-02	GGTGCGGGAA
33	OPE-03	CCAGATGCAC
34	OPE-04	GTGACATGCC
35	OPE-05	TCAGGGAGGT
36	OPE-06	AAGACCCCTC
37	OPE-07	AGATGCAGCC
38	OPE-08	TCACCACGGT
39	OPE-09	CTTCACCCGA
40	OPE-10	CACCAGGTGA

3.7.4.4 Agarose gel electrophoresis

To the 10 µl of amplification products obtained after the PCR reaction, 2 µl of loading dye was added and sample was loaded on 1.2% agrose (Amresco, USA) gel resolve using 1X TAE buffer, pre-stained with ethidium bromide (at1 µg/ml). 100 bp ladder (HiMedia, DNA ladder) was used as a marker. The gel was observed in a trans-illuminator over ultra violet light. Each amplification product was considered as RAPD marker and ISSR marker and recorded across all samples. The RAPD and ISSR pattern of each isolate was evaluated assigning character state 1 to all the bands that could be reproducibly detected on the gel and 0 for the absence of band at the same locus. Data was entered using a matrix in which all observed bands or characters were listed.

3.7.4.5 Estimation of genetic distance

The data matrix thus generated was used to calculate Jaccard's similarity co-efficient for pair wise comparisons. The coefficients were calculated *in silico* following Jaccard (1908), using following formula: $J = a / (n-d)$. Where, $\underline{a}$ is the number of positive matches (i.e., the number of bands present in both individuals), $\underline{d}$ is the number of negative matches (i.e., the number of bands absent in both individuals) and $\underline{n}$ is the total sample size including both the numbers of "match" and "unmatch". For this purpose, NTSYS-pc version 2.0 software (Rohlf, 1990) was used.

3.7.4.6 Cluster analysis

In the present study, cluster analysis was done using UPGMA (Unweighted Pair Group Method using arithmetic Averages) where similarity/dissimilarity (distance) between isolates in a cluster was established. After fusion of two most similar isolates, clustering continues between two next closest isolates or between any unplaced isolate and the established cluster. An unplaced isolate can join a cluster if its average similarity to all members of the cluster is small enough in comparison with any other pair of unplaced isolates. This process is repeated until all clusters join to form one cluster. This is an unweighted method because it gives equal weightage to each isolate within a cluster. The resultant similarity matrices based on Jaccard's measure were further analysed by performing sequential, agglomerative and hierarchical and nested clustering

(Rohlf, 1990) using the UPGMA method. The results of clustering were plotted in the form of dendrograms.

3.7.5 Internal Transcribed Spacer (ITS) region

A number of sequence based analysis was present to demonstrate the variation in nucleotide sequences, but it depends on the sequence chosen for analysis. The genomic DNA consists of the rDNA genes whose sequence data have been used to analyze major evolutionary events whereas the rDNA internal transcribed spacers, being less conserved, were employed successfully for investigating species-level relationships. Internal transcribed spacer regions in fungi have been found to evolve faster and therefore may contain sufficient nucleotide sequence variation to infer relationships between species (Fig. 3.1).

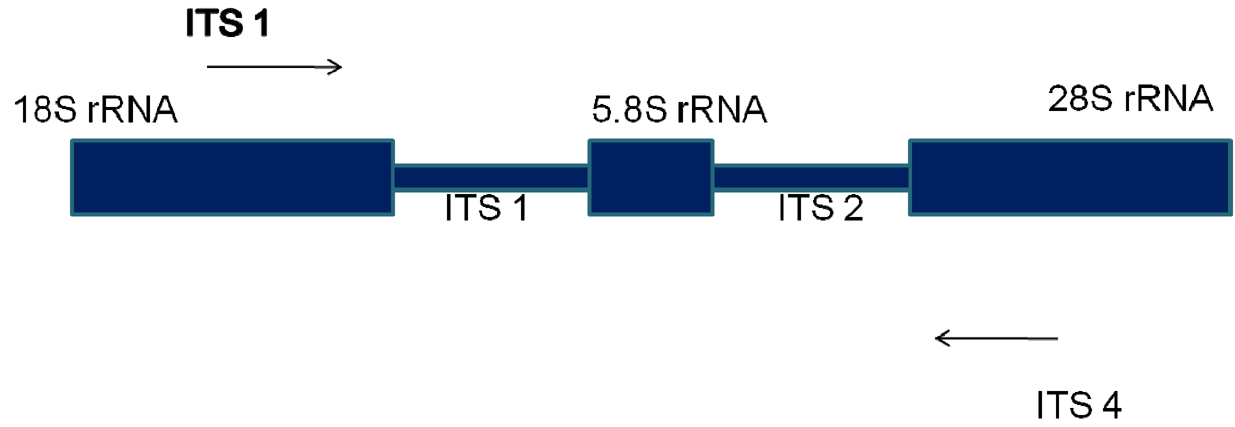


Fig. 3.1. Diagrammatic representation of rDNA- ITS region with ITS 1 & ITS 4 primers location

3.7.5.1 Specific amplification of ITS region by polymerase chain reaction

The regions of the rDNA repeat from the 3'end of the 18s and the 5' end of the 28s gene were amplified using PCR conditions with the two primers, ITS I and IV which were synthesized on the basis of conserved regions of the eukaryotic rRNA gene (White *et al*., 1990). The primer sequence and the region they span are represented in Table 3.4. The ITS regions and 5.8S rDNA of forty isolates of the pathogen representing various RAPD clusters were amplified. The PCR amplification reaction optimized for three concentrations of template DNA (25 ng, 50 ng, 75 ng) along with three concentrations of the $MgCl_2$ (50 µM, 100 µM, 200 µM). dNTP's mix (20 µM, 50 µM and 100 µM) and primer (1 µM, 2 µM and 3 µM) were used with one unit of *Taq* DNA polymerase and 10X reaction buffer (Fermantas life science, Canada). The final amplification was carried out in a using Biorad Gene Cycler^{TM} with 50µl reaction containing, DNA template 50 ng, $MgCl_2$ (200 µM), dNTP's mix (50 µM) and primers ITS 1 and ITS 2(2 µM each), one unit *Taq* DNA polymerase and 1X PCR buffer. Nuclease free water was used to bring the reaction volume to 50 µl. The cycle parameters included an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 1 min and primer extension at 72°C for 2 min and a final extension for 10 min at 72°C. Amplified products were separated on 1.3% agarose gel in TAE buffer, pre-stained with Ethidium bromide (1 µg/ml). One Kb ladder (MBI, Fermentas) was used as a marker. The gel was observed in a trans-illuminator over ultra violet light.

3.7.5.2 Sequence and phylogenetic relationships analysis

The BLAST program (Altschul *et al*., 1997) was used to identify and confirm the regions of the respective sequence. The nucleotide sequences were subjected to BLAST analysis on NCBI database (http://www.ncbi.nih.gov/blast.html). Sequences were submitted to NCBI *GenBank* and accession numbers were obtained. The multiple sequence alignment and pair wise alignment were made using BioEdit version 7.0.5 (Hall, 1999). Phylogentic tree was constructed based on the maximum likelihood of nucleotide sequences by using CLUSTALW 1.8 sequence alignment selecting Bootstrap neighboring joint by MEGA 4.1 programme.

Table 3.4 List of ITS primers used for PCR amplification of *A. brassicae* isolates

Primer name	Sequence (5’ - > 3’)
ITS – I	TCCGTAGGTGAACCTGCGG
ITS – IV	TCCTCCGCTTATTGATATGC

Table 3.5 Details of the genotypes used in the field study

Sl. No.	Crop	Variety
1	Cabbage	Golden Acre
		CJ 182
2	Cauliflower	Pusa Sharad
		Pusa Meghna
3	Mustard	Pusa Bold
		Pusa Jaganath

3.8 Role of weather in disease development;

3.8.1 Studies under Field conditions

Field trials at IARI, New Delhi (28o 36'N; 77o 12'E) were laid out in 2010-2011 and 2011-2012, rabi crop season in a randomized block design (RBD). In this study two varieties of cabbage, cauliflower and mustard were used along with mixed plot. The details of the genotypes used were given Table 3.5.

3.8.2 Land preparation

Field was prepared 15 days prior to sowing and weeds, stubbles and plant debris were removed. Soil was pulverized by two successive digging at the time of sowing. Separate nursery was used for seedling rise in case of cabbage and cauliflower.

3.8.3 Field layout

During 2010-11 and 2011-12, six genotypes were sown in field. The plot size of 3x1 m in randomized block design with 3 replications. Here two cultivars of mustard, cabbage and cauliflower each along with one mixed plot were raised.

3.8.4 Sowing/ transplanting

Sowing was done manually by keeping row to row distance 30 cm in mustard. Cabbage and cauliflower seedlings were transplanted manually at row to row distance of 60 cm. In case of cauliflower and cabbage the nursery was raised on 10th September 2010 and 2011. The transplanting was carried out on 15th October 2010 and 2011. Whereas, the mustard was directly sown on the respective plot.

3.8.5 Intercultural operations

Plants were thinned out to keeping 10 cm distances among the mustard, after one week of germination. Gap filling was carried out in case cabbage and cauliflower to maintain the effective plant population. Irrigation and weeding were done on time to time, whenever required. Recommended dose of fertilizer nitrogen, phosphorus and potash were given in the form of urea,

single superphosphate and muriate of potash respectively. Fertilizer were broadcasted and mixed thoroughly by light harrowing. Total amount of phosphorus and potash and 2/3 of the nitrogen were applied as basal dose and the remaining nitrogen was applied at two split doses during first and second irrigation.

3.8.6 Observation on disease component

Data for initial date of appearance of Alternaria blight and gradual progress of the same on leaves of cabbage, cauliflower and mustard were monitored. Observations for Per Cent Disease Index (PDI) were recorded once in five days from December to February. Ten randomly selected plants in each plot in each year were tagged for taking observations for disease component. There was a considerable variation in leaf size of different test genotype; therefore, for comparative study, per cent of diseased leaf were counted at five days interval. Observations were taken on ten plants per plots, which were tagged earlier.

The plants were evaluated individually giving 0-5 rating scale based on leaf area covered by spots considering necrotic as well as chlorotic areas.

The following plant disease index was calculated by using the following formula:

$$\text{Per cent Disease Index (PDI)} = \frac{\text{Sum of individual ratings}}{\text{Total number of plants/ leaves observed}} \times \frac{100}{\text{Maximum disease grade}}$$

Over all disease scoring at 0-5 rating scale was also done based on a disease assessment key for Alternaria black spot in crucifers developed by Wheeler (1969).

3.8.7 Plotting disease progressive curve (DPC):

For this purpose, interval were taken on X-axis and percent of disease index was taken on Y-axis, then the disease progressive curve for each genotype was plotted in respect of cabbage, cauliflower and mustard.

3.8.8 Area under disease progress curve (AUDPC)

AUDPC was computed using the formula of Wilcoxson *et al.* (1975), who quantified the AUDPC as A-value:

$$\text{AUDPC} = \sum_{i=1}^{k} \frac{1}{2} 1 (y_i + y_{i-1}) \times d$$

Where y_i is the disease incidence at i^{th} day of evaluation, k is the number of successive evaluation and d is the interval between i and i-1 evaluation of the disease.

3.8.9 Apparent rate of infection (r value):

The apparent rate of disease development (r) is a measure of the speed at which an epidemic develops. Despite the presence of virulent pathogen and favourable environment, differences were observed in the rate of disease development on different dates of sowing and two varieties culminating in low terminal disease severity. Apparent infection rates (r) were calculated from the Alternaria blight severity recorded at five days interval using the formula,

$$r = \frac{2.3}{t_2 - t_1} \left\{ \log \frac{x_1}{x_2} \right\}$$

Where 'r' is the apparent infection rate in non-logarithmic phase, x_1 is the disease index at initial week time (t_1), x_2 is the disease index at subsequent week time (t_2). The apparent infection rates were further used to assess the highest and least infection periods with respect to each crop cultivars and compared with local weather factors.

3.8.10 Determinations of optimum temperature for spore germinations

Spore germination was studied by hanging drop technique putting the spore in distilled sterilised water in cavity slides. The standard spore suspension containing 5 to 10 spores per microscopic field under low power was invariably used in each experiment. Cavity slides were then placed in a moist chamber prepared with the help of sterilized petriplates and incubated at 29°C (±2). Spores with germ tube longer than their width were taken to be as germinating. Germination of conidia generally started within four hours of their placement in water. This aspect was undertaken to assess the influence of different temperature (5, 10, 15, 17, 19, 21, 23, 25, 27, 29 and 31°C) on spore germination of the pathogen. Observations were recorded after 24 hr of inoculation. Average per cent germination was calculated and analysed statistically.

4. RESULTS 4.1. RESEARCH PAPER-I

Role of weather on Alternaria leaf spot development in crucifers.

Abstract

Black leaf spot caused by *Alternaria brassicae* (Berk.) Sacc. is an important disease of crucifers. Progression of Alternaria leaf spot was found to be influenced by environmental factors prevalent under field conditions. Disease progression was monitored once in five days on two chosen varieties of cabbage, cauliflower and mustard each. The pooled data for both years (2010-11 and 2011-12) revealed that there was periodical increase in lesion number and Per cent Disease Index (PDI). However, PDI increase varies with crops and crop varieties. PDI progression was higher in mustard varieties followed by cauliflower and cabbage varieties. The speed of progression of disease among the crop cultivars was calculated by using Area Under Disease Progress Curve (AUDPC) and apparent rate of infection (*r*-value). Highest AUDPC value was recorded on Pusa Bold variety of mustard and the lowest was recorded on CJ-182 variety of cabbage. This may enables to select the slow disease progressing varieties for the management of the disease. Relative humidity (morning and evening) were found negatively correlated with the development of disease. On the contrary, maximum and minimum temperature was positively correlated with disease development. But average temperature showed high degree of correlation than the minimum and maximum temperature. The laboratory study indicates the optimum conditions for spore germination was 20-24°C with more than 90 per cent relative humidity.

Key words:

Alternaria brassicae; Alternaria leaf spot; PDI; AUDPC; *r*-value

Introduction

Brassicaceae is an economically important family of flowering plants which gives oil yielding plant and vegetables. Pests and disease are the major constrains in the production of crucifers. Among the foliar diseases of crucifers, black leaf spot disease is one of the most destructive in nature. Black leaf spot caused by *Alternaria brassicae* (Berk.) Sacc. is an important disease of crucifers (cauliflower, cabbage, mustard, etc.) in the Indian sub-continent (Ansari *et al.,* 1988 and 1989). The disease appears annually during the cropping season (from October to February) in different parts of India and causes enormous loss to growers (Prasad *et al.,* 2006). The leaf spot phase reduces plant vigor and renders the crop unusable where leaves are the edible portion of the plant. Besides the leaf spot phase, edible flower parts of broccoli heads, cauliflower curds, seedlings, and flower parts related to seed production are also affected. In India, the leaf spot phase is the most common aspect of these diseases. In India, cabbage, cauliflower, mustards broccoli and turnips are infected commonly (Shrestha and Shrestha, 1992) and losses up to of 15 to 71 per cent was reported (Kadian and Saharan 1983; Singh and Bhowmik 1985; Kumar, 1986; Ram and Chauhan 1998). Environmental factors play an important role in the development of the disease. The environmental variables *viz.,* temperature, humidity, rainfall and sunshine are the most critical factors. Since they affect the pathogen and host or host pathogen interaction during pathogenesis. Temperature is one of the most important atmospheric factors which influence spore germination of parasitic fungi on aerial parts of plants. Several authors (Dey, 1948; Louvet, 1958; Louvet and Billotte, 1964) have found that leaf spot disease of crucifers caused by *A. brassicae* is favoured by low temperature, high humidity and splashing rain. Gupta *et al.* (1972) had earlier shown that alternate periods of darkness and light favours conidial germination in *A. brassicae*. Inhibition effects of leaf exudates and extracts, particularly of the resistant cultivars, to conidial germination would suggest the possibility of presence of some principle(s) in different *Brassica* sp. which might participate in natural defence. Moderate but prolonged incidence of *Alternaria* blight was observed in October sown crop and in November sown crop, whereas higher severity of Alternaria blight was observed in short duration crop (Singh *et al.,* 2009). The disease incidence and severity of Alternaria blight varies with crops and crop stages (Meena *et al.,* 2011). Information on influence of weather factors on development of disease can help in further refining disease management strategies (Dang *et al.,* 1995; Singh *et al.*, 1998). Thus, one of the major challenges of research on crucifers

is to develop knowledge about the epidemiological factors responsible for the development of Alternaria blight, ways to minimise the losses caused by these diseases. Kumar and Kolte (2001) have used the Area Under Disease Progress Curve (AUDPC) to demonstrating a slow blight progress in different cultivars compared to others. In the present study, the optimum temperature of spore germination and other weather factors for disease development in cabbage, cauliflower and mustard was studied.

Material and methods:

Field trials at Indian Agricultural Research Institute (IARI), New Delhi (28° 36'N; 77° 12'E) were laid out in 2010-2011 and 2011-2012, Rabi crop season in a randomized block design (RBD). In this study two varieties of cabbage (Golden Acre and CJ-182), cauliflower (Pusa Sharad and Pusa Meghna) and mustard (Pusa Bold and Pusa Jaganath) were used along with mixed plot. Sowing/transplanting was carried out at 15th October in each year with standard spacing and recommended doses of N, P and K fertilizer were applied. No protection measures were taken against any diseases.

Data for initial date of appearance of Alternaria blight and gradual progress of the same on leaves of cabbage, cauliflower and mustard were monitored. Observations for Per Cent Disease Index (PDI) were recorded once in five days from December to February. Ten randomly selected plants in each plot in each year were tagged for taking observations for disease component. There was a considerable variation in leaf size of different test genotype; therefore, for comparative study, per cent of diseased leaf were counted at five days interval. Observations were taken on ten plants per plots, which were tagged earlier. Over all disease scoring at 0-5 rating scale was also done based on a disease assessment key for Alternaria black spot in crucifers developed by Wheeler (1969). Per cent Disease Index was calculated using the following formula,

$$\text{Per cent Disease Index (PDI)} = \frac{\text{Sum of individual ratings}}{\text{Total number of plants/}} \times \frac{100}{\text{Maximum disease grade}}$$

leaves observed

AUDPC was computed using the formula of Wilcoxson *et al.* (1975), who quantified the AUDPC as A-value:

$$\text{AUDPC} = \sum_{i=1}^{k} \frac{1}{2} 1(y_i + y_{i-1}) \times d$$

Where y_i is the disease incidence at i^{th} day of evaluation, k is the number of successive evaluation and d is the interval between i and i-1 evaluation of the disease. The apparent rate of disease development (r) is a measure of the speed at which an epidemic develops. Despite the presence of virulent pathogen and favourable environment, differences were observed in the rate of disease development on different dates of sowing and two varieties culminating in low terminal disease severity. Apparent infection rates (r) were calculated from the Alternaria blight severity recorded at five days interval using the formula,

$$r = \frac{2.3}{t_2 - t_1} \left\{ \log \frac{x_1}{x_2} \right\}$$

Where 'r' is the apparent infection rate in non-logarithmic phase, x_1 is the disease index at initial week time (t_1), x_2 is the disease index at subsequent week time (t_2). The apparent infection rates were further used to assess the highest and least infection periods with respect to each crop cultivars and compared with local weather factors.

Spore germination was studied by hanging drop technique, in which spores were placed in sterilized distilled water in cavity slides. The standard spore suspension containing 5 to 10 spores per microscopic field under low power magnification (10X) was invariably used in each experiment. Cavity slides were then placed in a moist chamber prepared with the help of sterilized petriplates and incubated at different temperatures. Spore germination was observed periodically and spores with germ tube longer than their width were considered to be germinating. Germination of conidia generally started within four hours of their placement in water. This study was undertaken to assess the influence of different temperatures (5, 10, 15, 17,

19, 21, 23, 25, 27, 29 and 31°C) and Relative Humidity (RH) (84, 86, 88, 90, 92, 94, 96, 98 and 100 per cent) on spore germination of the pathogen. Observations were recorded after 24 hr of inoculation. Average per cent germination was calculated and analysed statistically.

Results:

At IARI field, during December- February, maximum temperature was 10-28°C, minimum temperature 1-15°C, relative humidity >80% (Figure 4.1.1). In both years, Alternaria leaf blight incidence was observed to be low. All the plots were daily examined for first appearance of spot on leaves and when the spot appeared in form of pin head, the date of appearance was recorded. In both the years Pusa Bold showed earliest occurrence of the symptoms on cotyledonary leaves of few plants during first week of November. However, the disease did not progress further till late December.

Significant difference among genotypes and time intervals were observed for all the components of disease resistance *viz.,* number of spots, size of spots, number of conidia per spot and disease index. Percent Disease Index (PDI) and AUDPC for Alternaria blight severity on leaves of different cultivars of cabbage, cauliflower and mustard were analysed (Table 4.1.1). Per cent blight severity on leaves of Pusa Jaganath was higher by 13.787 per cent and 15.242 per cent in 2010-2011 and 2011-2012 respectively. Whereas, lowest blight severity on leaves of Golden Acre 2.564 per cent and 2.347 per cent in 2010-2011 and 2011-2012 respectively. Among the cabbage cultivars Golden Acre showed lesser disease severity than the CJ-182 in both the year. In cauliflower, Pusa Sharad showed lesser disease severity than the Pusa Meghna in both years. Among the mustard variety Pusa Jaganath showed higher disease severity than Pusa Bold. In mustard both variety showed higher disease severity in 2011-12 than the 2010-2011(Fig. 4.1.3 and Fig. 4.1.4). Disease severity of the mixed plot is moderate *i.e.* higher than the cabbage cultivars but lesser than the mustard and cauliflower cultivars.

AUDPC (A-value) was observed decreasing order from mustard to cabbage (Table 4.1.2). The highest amount of A-value was observed in Pusa Bold (499.43) in 2011-2012. Lowest amount was observed in CJ 182 (102.57) in 2010-2011. Moreover, when compared to 2010-2011, 2011-2012 recorded highest A-value in all the cultivars except mixed plot containing the varieties of Golden Acre, Pusa Shard and Pusa Bold. The *r*-value was calculated from per cent

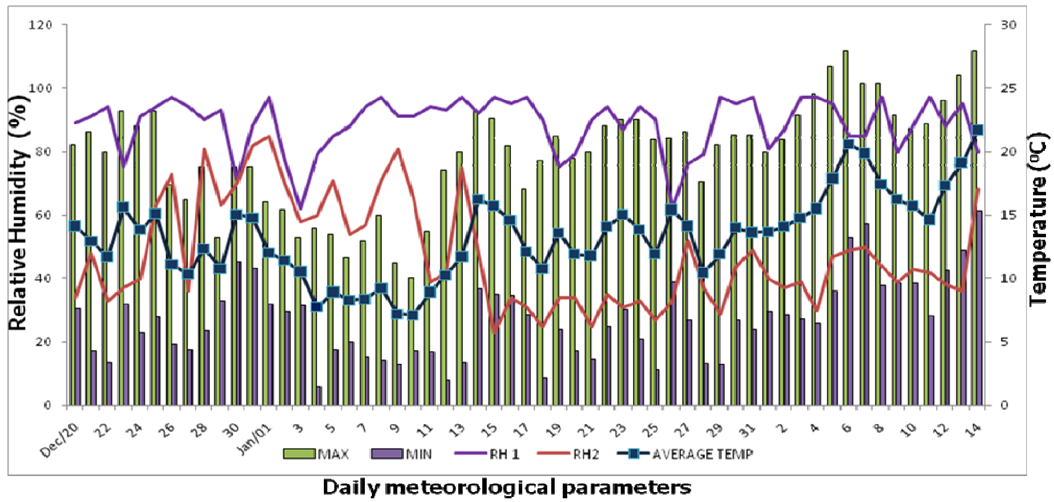


Fig. 4.1.1 Weather parameters during the crop season 2010-2011 at IARI, NewDelhi

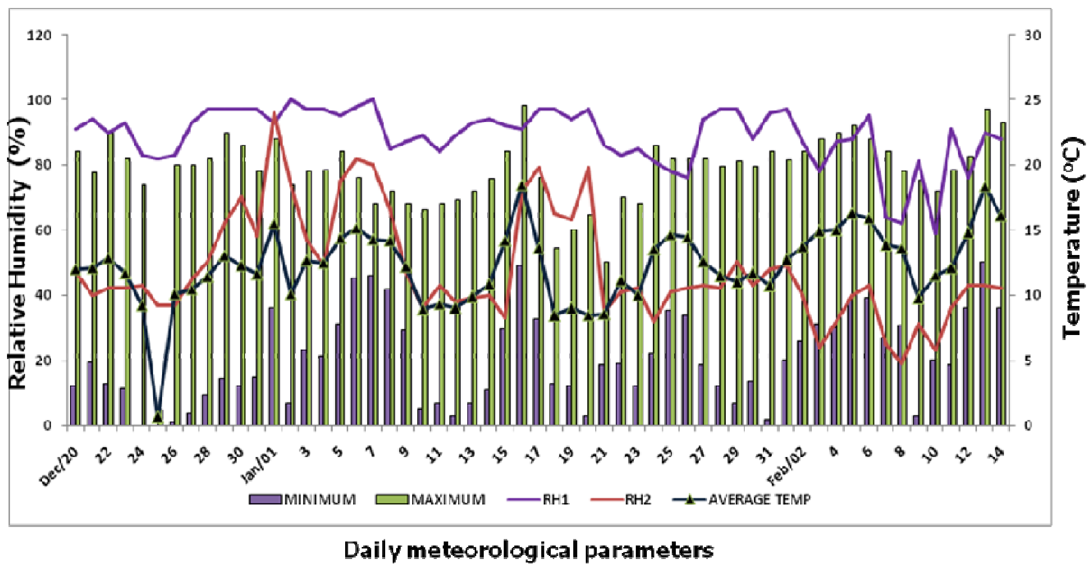


Fig. 4.1.2 Weather parameters during the crop season 2011-2012 at IARI, New Delhi

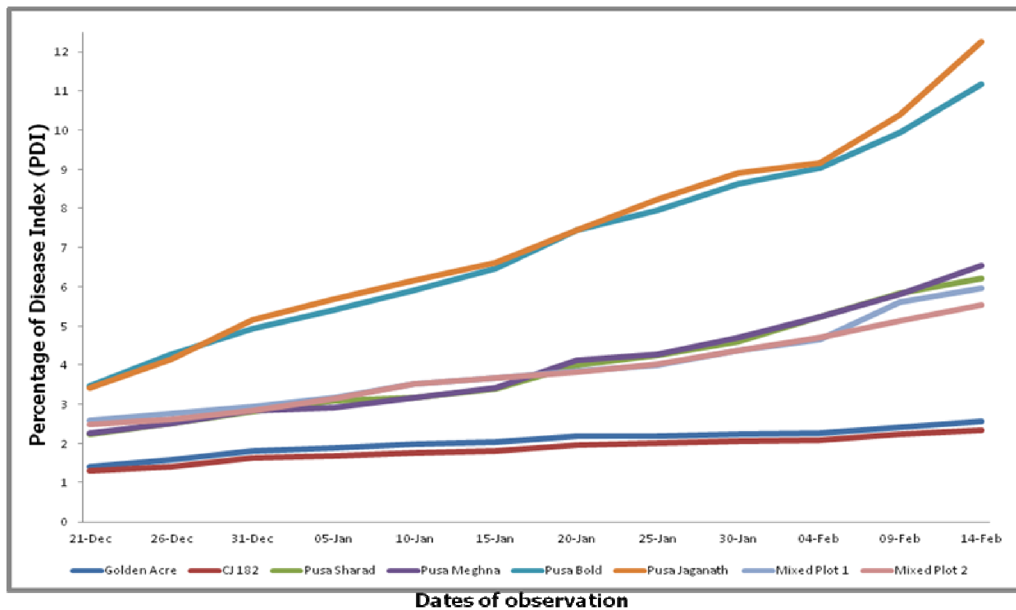


Fig. 4.1.3 Alternaria leaf spot disease progression among the cultivars during 2010-2011

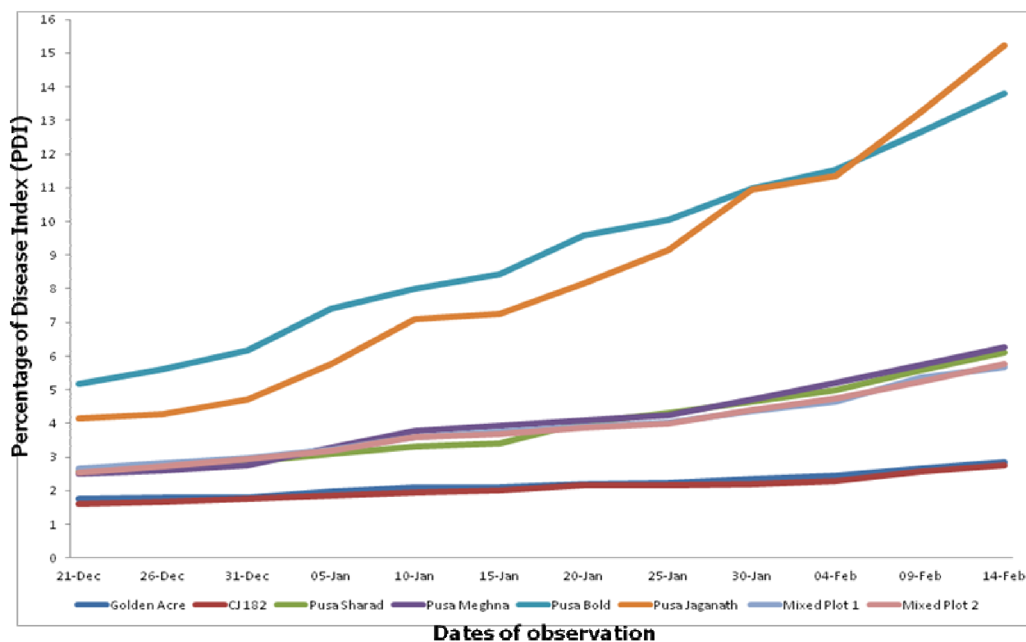


Fig. 4.1.3 Alternaria leaf spot disease progression among the cultivars during 2011-2012

Table 4.1.1 Per cent severity of Alternaria blight on cabbage, cauliflower, mustard and mixed plots.

DATE	Cabbage				Cauliflower				Mustard				Mixed plot			
	Golden Acre		CJ 182		Pusa Sharad		Pusa Meghna		Pusa Bold		Pusa Jaganath		Variety 1		Variety 2	
	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12
21-12	1.400	1.762	1.317	1.617	2.239	2.565	2.257	2.517	3.466	5.165	3.417	4.165	2.596	2.664	2.505	2.530
26-12	1.590	1.785	1.412	1.655	2.519	2.695	2.509	2.587	4.269	5.617	4.162	4.265	2.772	2.815	2.625	2.724
31-12	1.818	1.803	1.638	1.750	2.809	2.841	2.833	2.751	4.941	6.158	5.165	4.712	2.935	2.976	2.852	2.935
05-01	1.891	1.977	1.694	1.866	3.097	3.110	2.916	3.291	5.415	7.402	5.699	5.765	3.167	3.214	3.142	3.197
10-01	1.995	2.095	1.752	1.960	3.176	3.325	3.165	3.769	5.915	8.005	6.162	7.107	3.531	3.607	3.528	3.600
15-01	2.032	2.117	1.812	2.014	3.387	3.416	3.417	3.927	6.462	8.432	6.616	7.265	3.687	3.736	3.678	3.702
20-01	2.180	2.196	1.973	2.156	4.003	4.024	4.119	4.100	7.447	9.592	7.457	8.165	3.842	3.898	3.825	3.869
25-01	2.183	2.242	2.012	2.172	4.256	4.298	4.270	4.248	7.964	10.033	8.226	9.156	4.007	4.017	4.020	3.985
30-01	2.241	2.362	2.058	2.196	4.599	4.656	4.716	4.720	8.641	10.983	8.915	10.933	4.387	4.384	4.391	4.401
04-02	2.256	2.456	2.082	2.295	5.235	4.982	5.228	5.213	9.029	11.523	9.152	11.358	4.663	4.664	4.702	4.736
09-02	2.413	2.665	2.252	2.563	5.848	5.565	5.816	5.726	9.941	12.665	10.401	13.256	5.603	5.349	5.141	5.226
14-02	2.564	2.847	2.347	2.765	6.216	6.097	6.556	6.251	11.165	13.787	12.266	15.242	5.967	5.666	5.542	5.754
CD (0.05)	0.0965	0.0895	0.0963	0.0789	0.956	0.895	0.0895	1.058	0.569	0.689	0.895	1.236	0.456	0.421	0.564	0.236

Table 4.1.2 AUDPC (A-VALUE) for severity of Alternaria blight on cabbage, cauliflower, mustard and mixed plots.

Crops		2010-11 (mm^2)	2011-12 (mm^2)
Cabbage	Golden Acre	112.91	120.00
	CJ 182	102.57	114.09
Cauliflower	Pusa Sharad	215.78	216.21
	Pusa Meghna	216.97	223.57
Mustard	Pusa Bold	386.70	499.43
	Pusa Jaganath	398.98	458.42
Mixed plot	Variety 1	214.38	214.13
	Variety 2	209.63	212.59
CD (0.05)		5.658	5.697

disease index (Table 4.1.3). It was varying from 0 to 0.020. The highest *r*-value was recorded in Pusa Jaganath cultivar of mustard between 26-12-2010 to 31-12-2010. The lowest *r*-value was recorded in cabbage cultivar Golden Acre between 20-01-2011 to 25-01-2011.

Correlation analyses of disease index with weather factors indicated that maximum temperature (10-28°C) and minimum temperature (1.5-15.3^{o}C) has significant positive correlation with disease index. Average temperature also showed the positive correlation, which is higher than the minimum and maximum temperature. While morning relative humidity not has significant correlation but the evening relative humidity has a significant correlation (Table 4.1.4).

In laboratory condition *Alternaria brassicae* responded variably to different temperature treatments. A gradual increase in the temperature from 5°C enhanced spore germination up to 24°C beyond which germination declined drastically (Table 4.1.5). In 24°C the spore germination was higher followed by 22°C, 20°C, 26°C, 15°C, 28°C and 10°C. There was negligible spore germination taken place in 0°C, 5°C, 30°C, 35°C and 40°C. Spore germination at all the levels of relative humidity was tested. Spore germination percentage was increasing with relative humidity and showed higher correlation coefficient (0.955). Highest spore germination percentage was seen at 100 per cent relative humidity.

Discussion:

Severity of Alternaria blight was significantly influenced by weather factors like minimum temperature, maximum temperature, relative humidity etc. In present study a relationship between atmosphere temperature and relative humidity and infection and disease progression was studied. The progress of the disease was found to be highly varied among the crops and cultivars. Gupta *et al.* (2003) tested three varieties of mustard against Alternaria blight and found that significant variation in disease index. Maximum temperature showed positive correlation with Alternaria blight severity and minimum temperature did not showed higher correlation, which is not matching with earlier findings of Awasthi and Kolte (1994). Singh *et al.* (2009) also found the positive correlation between maximum, minimum temperature and disease index. But Meena *et al.* (2011) found that maximum temperature positively influenced Alternaria blight severity and minimum temperature had less influence. In 2010-2011, the influence of

Table 4.1.3 Apparent infection rate (*r*-value) of Alternaria blight on cabbage, cauliflower, mustard and mixed plots.

Date	Cabbage				Cauliflower				Mustard				Mixed plot			
	Golden Acre		CJ 182		Pusa Sharad		Pusa Meghna		Pusa Bold		Pusa Jaganath		Variety 1		Variety 2	
	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12	2010-11	2011-12
26-12	0.012	0.001	0.006	0.002	0.011	0.005	0.010	0.003	0.019	0.008	0.018	0.002	0.006	0.005	0.004	0.007
31-12	0.012	0.001	0.014	0.005	0.010	0.005	0.011	0.006	0.013	0.008	0.020	0.009	0.005	0.005	0.008	0.007
05-01	0.004	0.008	0.003	0.006	0.009	0.008	0.003	0.016	0.008	0.017	0.009	0.019	0.007	0.007	0.009	0.008
10-01	0.005	0.005	0.003	0.005	0.002	0.006	0.008	0.012	0.008	0.007	0.007	0.019	0.010	0.011	0.011	0.011
15-01	0.002	0.001	0.003	0.002	0.006	0.002	0.007	0.004	0.008	0.005	0.007	0.002	0.004	0.003	0.004	0.003
20-01	0.006	0.003	0.008	0.006	0.015	0.015	0.017	0.004	0.013	0.012	0.011	0.011	0.004	0.004	0.004	0.004
25-01	0.000	0.002	0.002	0.001	0.006	0.006	0.003	0.003	0.006	0.004	0.009	0.011	0.004	0.003	0.005	0.003
30-01	0.002	0.005	0.002	0.001	0.007	0.007	0.009	0.010	0.007	0.008	0.007	0.016	0.008	0.008	0.008	0.009
04-02	0.001	0.004	0.001	0.004	0.012	0.006	0.009	0.009	0.004	0.004	0.002	0.004	0.006	0.006	0.006	0.007
09-02	0.006	0.008	0.007	0.010	0.010	0.010	0.010	0.009	0.009	0.009	0.012	0.014	0.017	0.013	0.008	0.009
14-02	0.006	0.006	0.004	0.007	0.006	0.008	0.011	0.008	0.011	0.008	0.015	0.013	0.006	0.005	0.007	0.009

Table 4.1.4 Correlation coefficient between weather factors and Alternaria blight disease index.

Crops		2010-11					2011-12				
		Relative humidity 1	Relative humidity 2	Maximum temperature	Minimum temperature	Average temperature	Relative humidity 1	Relative humidity 2	Maximum temperature	Minimum temperature	Average temperature
Cabbage	**Golden Acre**	0.070	-0.425	0.463	0.369	0.482	-0.457	-0.398	0.2419	0.366	0.361
	CJ 182	0.063	-0.450	0.511	0.388	0.523	-0.456	-0.385	0.236	0.357	0.354
Cauliflo wer	**Pusa Sharad**	-0.011	-0.437	0.292	0.159	0.269	-0.466	-0.429	0.253	0.325	0.345
	Pusa Meghna	0.076	-0.470	0.615	0.445	0.620	-0.445	-0.400	0.248	0.357	0.360
Mustard	**Pusa Bold**	0.063	-0.484	0.576	0.399	0.572	-0.434	-0.388	0.243	0.346	0.351
	Pusa Jaganath	0.055	-0.444	0.556	0.416	0.566	-0.465	-0.421	0.250	0.341	0.352
Mixed plot	**Variety 1**	0.092	-0.396	0.585	0.456	0.604	-0.502	-0.426	0.255	0.353	0.362
	Variety 2	0.083	-0.422	0.545	0.399	0.550	-0.501	-0.414	0.256	0.348	0.360

Table 4.1.5 Percentage germination of spores of *Alternaria brassicae* after incubation at different temperature

Temperature (^oC)	Percentage germination
0	0
5	0.410
10	10.975
15	35.675
20	82.35
22	91.35
24	93.6
26	57.525
28	13.475
30	1.0
35	0.05
40	0.05
CD (0.05)	0.3029

maximum temperature in Alternaria blight development was also varied between crop and season. The correlation between the maximum temperature and Alternaria blight was higher in case of 2010-2011. And the influence of minimum temperature was lower and no significant difference was seen between 2010-2011 and 2011-2012. Morning (maximum) RH has less influence on Alternaria blight severity with its correlation coefficient ranges from 0.09 to -0.01 in 2010-2011. But in 2011-2012 it ranges from -0.5 to -0.43. So the influence of morning relative humidity varies with the weather and cultivar used. In both the years, the influence of afternoon relative humidity influences Alternaria blight severity. Its correlation coefficient ranges from -0.38 to -0.44. Singh *et al.* (2009) also recorded negative correlation between disease index and weather factors like relative humidity I and II. In present study mustard shown higher susceptibility and followed by cauliflower and cabbage. This is clearly indicates that not only the weather parameter but also physiological and biochemical composition of plant as well as plant age plays a major role in plant disease development (Meena *et al*. 2004).

The Alternaria blight development highly depends on spore germination and incubation period. Nowicki *et al.* (2012) reported that the optimal spore germination temperature ranges from 18-24°C and relative humidity >90 per cent. In the present study also the optimal spore germination temperature for *A. brassicae* was recorded as 20-24°C, although spores could germinate at a wide range of temperature (10-30°C). This shows that the pathogen has adaptability to wide range of temperature. High air humidity (90-100 % RH) is a critical for the *A. brassicae* infection (Humpherson-Jones and Phelps, 1989). In the present study, *A. brassicae* spores germinated at all levels of relative humidity tested. But higher levels were more favorable (Table 4.1.6). The present study indicated the influence of weather factors on disease development. The correlation coefficient showed that there might be some other factor(s) involved in the disease development, which requires further studies. This information becomes a useful guide for the selection of crops and crop cultivars among crucifers and efficient management of pathogen.

Table 4.1.6 Percentage germination of spores of *Alternaria brassicae* after incubation at different relative humidity

Relative humidity (%)	**Percentage germination**
84	61.75
86	72.45
88	78.525
90	82.05
92	84.30
94	91.35
96	93.6
98	94.4
100	95.00
CD (0.05)	0.2972

4.2. RESEARCH PAPER II

Morphological and pathogenic diversity of *Alternaria brassicae* (Berk.) Sacc. isolates causing disease of black leaf spot of Crucifers.

Abstract

Alternaria brassicae is the most virulent pathogen on all brassicaceous plants and cause adverse effect on both quality and quantity. Present investigation was carried out to know the cultural, morphological and pathogenic variability in *A. brassicae* causing black leaf spot. Forty isolates of *A. brassicae* were collected from different cauliflower, cabbage and mustard growing locations of India and characterized for cultural, morphological, pathogenic variations. All the isolates showed high level of variability *in vitro* in respect to mycelia growth, growth pattern and sporulation. The Jaipur isolate (AB-16) showed higher growth (86 mm) in Potato Dextrose Agar (PDA) and Coimbatore isolate (AB-37) showed least growth (35mm) in PDA. All the isolates depicted high growth rate and high number of spore production on Cauliflower Leaf Extract Agar followed by Potato Dextrose Agar and Czepak Dox Agar. Substantial variation was found in spore morphology in respect to conidial length, width and number of septa. Average conidial length and width were varied from 34.2-99.2 µm and 6.1-14.8 µm respectively. Horizontal septa showed higher degree of variation than vertical septa. Number of horizontal and vertical septa ranged between 4.0-8.2 and 0.2 -1.8 respectively. All the forty isolates were tested on fifteen varieties, five each in cabbage, cauliflower and mustard by detached leaf technique. It revealed that all these isolates behaved differently on differentials. The Per Cent Disease Index (PDI) varied from 0-100 per cent. Delhi isolate AB-12 appeared to be more virulent infecting all differential. The studies, therefore, indicated the existence of variability among isolates of *Alternaria brassicae*.

Key Words:

Black leaf spot; Morphological variability; Pathogenic variability; Per cent disease index

Introduction

Brassicaceae (crucifer) is an economically important family of flowering plants which gives oil yielding plant and vegetables. Pests and disease are the important constrains in the production of crucifers, among the foliar diseases of crucifers, black leaf spot disease (*Alternaria brassicae*) is one of the most destructive diseases, commonly prevailing in almost all crucifer growing areas of the world. The yield losses in crucifers due to this disease under favorable conditions vary up to 80 per cent (Smith *et al.* 1988). Severity of black leaf spot disease caused by (*Alternaria brassicae*) on cruciferae differ among seasons and regions. This may be due to existence of variability within the isolates of *Alternaria* spp, this had been reported by various workers (Varma *et al*., 2006 and Goyal *et al.,* 2011). The necessity to boost resistance breeding programme accentuates the need in depth study of the diversity of *A. brassicae* throughout crucifer growing areas in the country. Variability in the morphological characteristics in *A. brassicae* isolates of different regions of India had been reported by early workers (Meena *et al.* 2005 and Singh *et al.* 2007). The taxonomy of *Alternaria* species is predominantly based on spore morphology, cultural characteristics and host pathogen association. Some report on cultural variability in *Alternaria brassicae* in respect to mycelial growth and sporulation on different temperature, relative humidity, hydrogen ion concentration, media and light (Ansari *et al*. 1989 and Patni *et al*. 2005). Kaur *et al*. (2007) also reported variability in *Alternaria brassicae* on the basis of morphology, sporulation, growth and other cultural characters. Different temperature were found optimum for growth and sporulation of *Alternaria brassicae* in a range of 20-25°C (Singh *et al*. 2007) and 20-30°C (Meena *et al.* 2005) respectively and Meena *et al.,* 2005 reported that mycelia growth, sporulation is also affected by relative humidity and showed variation in its requirement. Pathogenic variability of *A. brassicae* was observed by various workers (Varma and Saharan, 1994) however, information on existence of distinct pathotype using standard host differentials is rather limited (Saharan, 1992). Mridha, (1983) reported pathogenic variability in *A. brassicae* on selected cultivars of winter oilseed rape by analyzing pathogenic virulence pattern. Similarly Awasthi and Kolte (1989) also reported pathogenic variability in *A. brassicae*. None of the workers used different *Brassica* species to distinguish isolates of *A. brassicae* on the basis of their reaction on host differentials.

The purpose of this study was to examine cultural and morphological diversity among *Alternaria brassicae* isolates from different agro-climatic regions of India, to determine the

spatial distribution of this diversity and evaluate the pathogenic variability on the basis of their reactions to the genotypes of cauliflower, cabbage and mustard.

Material and methods:

Collection, identification and maintenance of isolates

Isolation and purification of *Alternaria brassicae* cultures was done from infected leaves of cruciferous plants like mustard, cauliflower and cabbage collected from different locations of India (Fig. 4.2.1). *A. brassicae* cultures were identified based on morphological characteristics. The cultures were purified by single spore isolation method and stored on PDA slants at 4º C (Table 4.2.1) for further study.

Cultural variability

Cultural characters such as colony colour, colony texture and radial growth were recorded on the seventh day of inoculation of all the *A. brassicae* isolates. Characters were recorded by direct observation of culture grown on Petriplates and sporulation was observed on three different media by microscope. All the 40 isolates of *A. brassicae* were grown and tested on three different media *i.e.* Potato Dextrose Agar (PDA), Czapek dox agar (CDA) and Cauliflower Leaf Extract Agar (CLEA) medium for cultural variability studies. The plates containing PDA, CDA and CLEA were inoculated centrally with 5 mm culture discs taken from periphery of 7 days old culture and incubated at 28±2°C. Observations on cultural characteristic *viz,* colony colour, type and growth were recorded a week after inoculation.

Morphological variability

The morphological characters of 40 isolates of *A. brassicae viz.,* size of conidia, beak length and number of transverse and longitudinal septa were recorded from 15 days old culture. The size of conidia was measured randomly from different microscopic fields under high power objective (40X) using ocular and stage micrometer. The data was analyzed statistically and average of 10 observations with standard error of mean was presented for each parameter.

Pathogenic variability

Pathogenic variation in the isolates of *A. brassicae* was ascertained on the basis of the ability of each of the isolates to cause diseases in fifteen different varieties of mustard, cabbage and cauliflower. The study was performed by detached leaf technique described by Sharma *et al.*, 2004. In this method third or fourth detached leaves from plants of mustard, cabbage and cauliflower grown in IARI field were used. Here area of approximately 3 mm^2 on the upper leaf

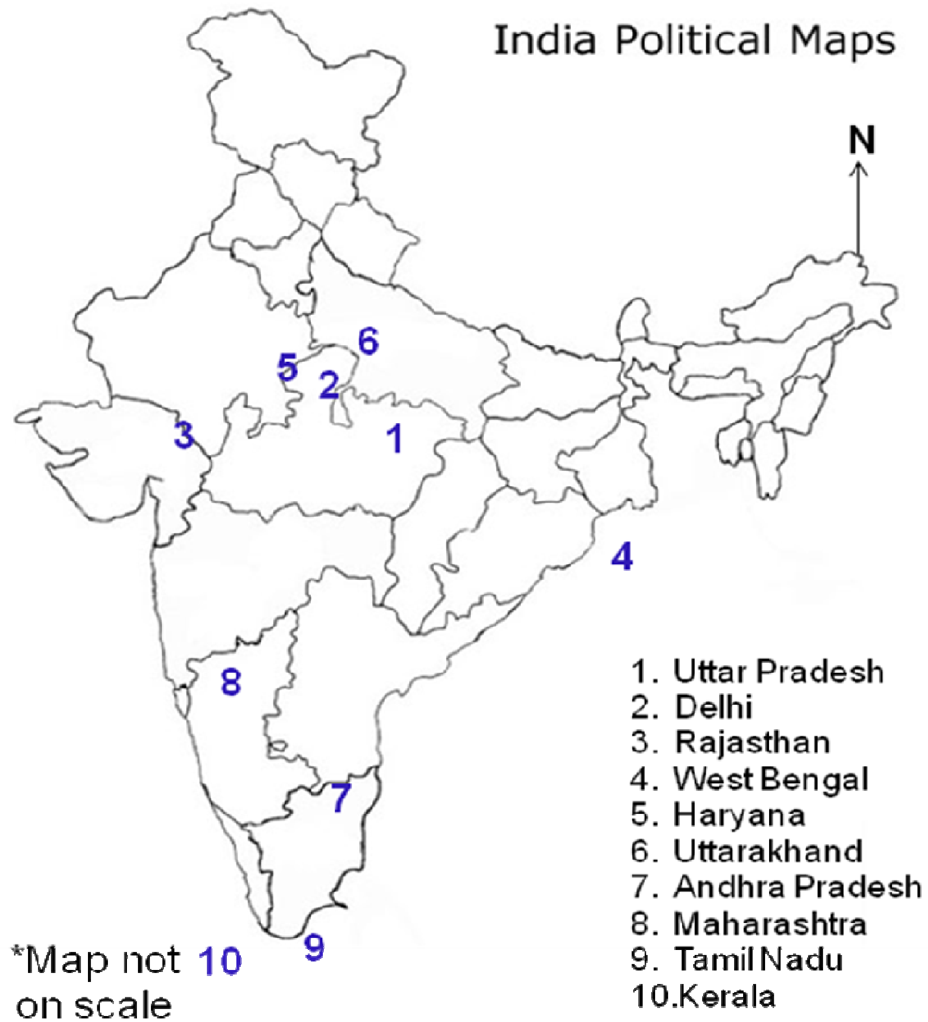


Fig. 4.2.1 India map showing locations from where isolates of *Alternaria brassicae* collected

Table 4.2.1 Details of *Alternaria brassicae* isolates used in the study

Sl. No.	Isolates Code	Host	Location	State	Accession Nos. of ITS sequences
1	AB-1	Cauliflower	Gaziabad	Uttar Pradesh	JF439438
2	AB-2	Cauliflower	Alipur	Uttar Pradesh	JF439439
3	AB-3	Cauliflower	Noida	Uttar Pradesh	JF439440
4	AB-4	Cauliflower	Meerut	Uttar Pradesh	JF439441
5	AB-5	Cauliflower	Bagpat	Uttar Pradesh	JF439442
6	AB-6	Cauliflower	Saharpur	Uttar Pradesh	JN108902
7	AB-7	Cauliflower	Kanpur	Uttar Pradesh	JF439443
8	AB-8	Cauliflower	Luknow	Uttar Pradesh	JF439444
9	AB-9	Mustard	Rampur	Uttar Pradesh	KF543032
10	AB-10	Mustard	IARI	Delhi	KF543033
11	AB-11	Cabbage	IARI	Delhi	KF543034
12	AB-12	Cauliflower	IARI	Delhi	JF439431
13	AB-13	Cauliflower	Najabgarh	Delhi	JF439432
14	AB-14	Cauliflower	Saraikalakhan	Delhi	JF439433
15	AB-15	Cauliflower	Mother dairy field	Delhi	JF439434
16	AB-16	Cauliflower	Jaipur	Rajasthan	JF439436
17	AB-17	Mustard	Baratpur	Rajasthan	KF543035
18	AB-18	Cauliflower	Kalyani	West Bengal	JF439448
19	AB-19	Cauliflower	Hoogli	West Bengal	JF439449
20	AB-20	Cauliflower	Kolkata	West Bengal	JF439450
21	AB-21	Cauliflower	Sonepet	Haryana	JF439445
22	AB-22	Mustard	Mewat	Haryana	KF543036
23	AB-23	Mustard	Hisar	Haryana	KF543037
24	AB-24	Cabbage	Gurgaon	Haryana	KF543038
25	AB-25	Mustard	Karnal	Haryana	KF543039
26	AB-26	Mustard	Pant nagar	Uttarakhand	KF543040
27	AB-27	Cabbage	Pant nagar	Uttarakhand	KF543041
28	AB-28	Cauliflower	Pant nagar	Uttarakhand	KF543042
29	AB-29	Cabbage	Nainital	Uttarakhand	KF543043
30	AB-30	Cauliflower	Nainital	Uttarakhand	KF543044
31	AB-31	Mustard	Kashipur	Uttarakhand	KF543045
32	AB-32	Cauliflower	Almora	Uttarakhand	KF543046
33	AB-33	Cabbage	Hyderabad	Andhra Pradesh	KF543047
34	AB-34	Cauliflower	Nagpur	Maharashtra	KF543048
35	AB-35	Cabbage	Dindugul	Tamil nadu	KF543049
36	AB-36	Cabbage	Palani	Tamil nadu	KF543050
37	AB-37	Cauliflower	Coimbatore	Tamil nadu	JF439446
38	AB-38	Cabbage	Theni	Tamil nadu	KF543051
39	AB-39	Cabbage	Hosur	Tamil nadu	KF543052
40	AB-40	Cauliflower	Palakad	Kerala	JF439447

surface was gently scratched and 1 ml of spore suspension (5 X 10^3 spores/ml) was injected by using sterilized syringe. The spore suspension was applied at six places on each leaf. The treated leaves were kept in polyethylene bags having moist blotting papers to maintain relative humidity (85-100%) at 27° C. The symptoms started appearing after fifth day of inoculation, symptoms rated visually by using 1-9 scale (1= no infection, 9=89-100% infection), plants rated 1-3 are resistant, 4-6 are moderately resistant and 7-9 as susceptible. Entries with > 75% susceptible plants are classified as susceptible, 50-74.9% as moderately susceptible, 25-49.9% as moderately resistance, 1-25% resistant and 0-0.99% as highly resistant.

Results

Pathogenicity test

Pathogenicity of *A. brassicae* isolates was proved by Koch's postulates by inoculating them on susceptible plants under laboratory condition. All the forty isolates cultures produced susceptible symptoms starting with small yellow and necrotic spots, 7 days after inoculation. Later, all spots enlarge with purple colour surrounded by yellow. The symptomatology of all the isolates was compared with the previous study.

Cultural variation

Various cultural characters like colony texture, colour and diameter were studied on PDA, CDA and CLEA. The data regarding various cultural characters are presented in (Table 4.2.2)

Colony growth

The data presented in Table 4.2.2 showed that there was significant variability among the isolates with regard to colony diameter. Isolates AB-16 registered the highest colony diameter of 78.7 mm average in three media tested (86 mm in PDA, 65 mm in CDA and 85 mm in CLEA), with the isolates AB-37 showed least colony diameter of 31.5 mm average (35 mm in PDA, 25 mm in CDA and 35 mm in CLEA). Based on the colony diameter of three different media the isolates were classified into three groups. The first group comprises of isolates (AB-10, AB-16, AB-17, AB-19, AB-21, AB-22, AB-26 and AB-28) which produced a colony of more than 70 mm diameter after seven days of inoculation on above mentioned medium. The second group comprises of isolates (AB-2, AB-3, AB-4, AB-5, AB-6, AB-8, AB-11, AB-12, AB-13, AB-14, AB-15, AB-18, AB-23, AB-24, AB-25, AB-27, AB-29, AB-30, AB-31, AB-32, AB-33, AB-34, AB-35 and AB-40) which produced a colony growth of 50-70 mm diameter in media. Remaining

Table 4.2.2. Colony diameter of *Alternaria brassicae* isolates on different media

Sl. No.	Isolates Code	Colony growth (mm) on media			
		PDA	CDA	CAM	Mean
1	AB-1	35	30	42	35.7
2	AB-2	60	58	76	64.7
3	AB-3	58	50	74	60.7
4	AB-4	62	57	72	63.7
5	AB-5	60	53	75	62.7
6	AB-6	60	54	73	62.3
7	AB-7	36	33	43	37.3
8	AB-8	52	50	68	56.7
9	AB-9	43	40	53	45.3
10	AB-10	72	69	78	73
11	AB-11	63	63	73	66.3
12	AB-12	71	68	70	69.7
13	AB-13	73	63	73	69.7
14	AB-14	66	63	66	65
15	AB-15	65	60	63	62.7
16	AB-16	86	65	85	78.7
17	AB-17	76	70	80	75.3
18	AB-18	63	63	64	63.3
19	AB-19	73	66	73	70.7
20	AB-20	46	46	53	48.3
21	AB-21	74	69	80	74.3
22	AB-22	70	71	71	70.7
23	AB-23	69	66	72	69
24	AB-24	55	50	62	55.7
25	AB-25	61	59	62	60.7
26	AB-26	73	71	74	72.7
27	AB-27	52	51	54	52.3
28	AB-28	70	69	73	70.7
29	AB-29	52	45	60	52.3
30	AB-30	51	46	59	52
31	AB-31	67	32	72	57
32	AB-32	71	54	79	68
33	AB-33	60	54	72	62
34	AB-34	52	47	60	53
35	AB-35	61	32	60	51
36	AB-36	55	29	65	49.7
37	AB-37	35	25	35	31.7
38	AB-38	48	25	50	41
39	AB-39	55	30	56	47
40	AB-40	72	42	80	64.7
CD (0.05)		12.69	8.95	13.55	

isolates formed third group (AB-1, AB-7, AB-9, AB-20, AB-36, AB-37, AB-38 and AB-39) which showed less than 50 mm diameter of colonies. Among the three media used for colony growth isolate AB-16 showed highest growth (86 mm) while, AB-1 and AB-37 showed the least (35 mm) growth. On Czapek Dox Agar media highest growth was seen in AB-22 and AB-26 (71 mm) isolates and least growth was seen in AB-37 and AB-38 (25 mm) isolates. Similarly, in Cauliflower Leaf Extract Agar highest growth was observed in AB-16 (85 mm) and least growth was observed in AB-37 (35 mm). The colour of the colonies grown on PDA varied between isolates. The colour of the *A. brassicae* isolates varied from whitish to blackish (Table 4.2.3). The isolates AB-1, AB-3, AB-4, AB-5, AB-7, AB-17 and AB-36 produced circular colonies and remaining isolates produced irregular colonies. Some isolates (AB-2, AB-4, AB-6, AB-8, AB-17, AB-20, AB-21, AB-22, AB-25, AB-28, AB-29, AB-30, AB-31, AB-33, AB-34, AB-35, AB-36, AB-39 and AB-40) produced clear zonation in the PDA (Fig. 4.2.2). Sporulation of each *A. brassicae* isolates on seventh day on different media was studied. The results are almost similar in different media. The sporulation potential ranges from 0.72 - 3.90 X 10^4/ml. The lowest sporulation was observed in AB-9 (0.72 X 10^4/ml) isolate and highest sporulation was observed in AB-18 (3.90 X 10^4/ml) isolate. Most of the isolates depicted high growth rate and high number of spore production on Cauliflower Leaf Extract Agar followed by PDA and CDA.

Spore Morphology

Morphological characters like length, width, septa and number of conidia in each of the forty isolates of *A. brassicae* were studied by microscopy and observations were made for each field. The data in the Table 4.2.4 showed a significant difference in length and width of conidia of *A.brassicae*. Average conidial length, varied from 34.2-99.2 µm, Tamil Nadu isolate (AB-38) showed highest conidial length (99.2 µm) and lowest length (34.2 µm) was seen in Hisar isolate (AB-23). Average conidial width varied from 6.1-14.8 µm, was highest was observed (14.8 µm) in Rampur isolate (AB-9) and lowest (6.1 µm) was observed in Lucknow isolate (AB-8). Finally it was revealed that the smallest size of conidia and lowest number of septa was seen in Hisar isolate (AB-23). Microscopic examination of conidia at 40X magnification revealed variability in conidia size and could be categorized into two groups, the first group comprises conidial length < 50 µm and second group comprises of long conidial length (>50 µm) but not according to their geographical origin. The following isolates comes under first group (<50 µm) AB-1, AB-2, AB-3, AB-4, AB-8, AB-13, AB-14, AB-15, AB-16, AB-20, AB-21, AB-23, AB-24, AB-25, AB-30,

Table 4.2.3. Growth characters of *Alternaria brassicae* isolates on Potato Dextrose Agar (PDA) medium

Sl. No.	Isolate Number	Colony colour	Mycelial growth/Colony character		
			Circular/ irregular	Smooth / rough	Zonation
1	AB-1	Olive Green	Circular	Smooth	No zonation
2	AB-2	Blackish	Irregular	Rough	Zonation
3	AB-3	Blackish	Circular	Rough	No zonation
4	AB-4	Dark gray	Circular	Smooth	Zonation
5	AB-5	Olive gray	Circular	Rough	No zonation
6	AB-6	Dark gray	Irregular	Rough	Zonation
7	AB-7	Olive gray	Circular	Smooth	No zonation
8	AB-8	Blackish	Irregular	Rough	Zonation
9	AB-9	Blackish	Irregular	Rough	No zonation
10	AB-10	Whitish Black	Irregular	Rough	No zonation
11	AB-11	Whitish	Irregular	Rough	No zonation
12	AB-12	Dark gray	Irregular	Rough	No zonation
13	AB-13	Black	Irregular	Rough	No zonation
14	AB-14	Dark gray	Irregular	Rough	No zonation
15	AB-15	Brownish black	Irregular	Rough	No zonation
16	AB-16	Grayish	Irregular	Smooth	No zonation
17	AB-17	Blackish	Circular	Rough	Zonation
18	AB-18	Dark gray	Irregular	Rough	No zonation
19	AB-19	Dark gray	Irregular	Rough	No zonation
20	AB-20	Blackish	Irregular	Rough	Zonation
21	AB-21	Dark Green	Irregular	Rough	Zonation
22	AB-22	Dark green	Irregular	Rough	Zonation
23	AB-23	Whitish	Irregular	Rough	No zonation
24	AB-24	Whitish	Irregular	Rough	No zonation
25	AB-25	Whitish Green	Irregular	Rough	Zonation
26	AB-26	Dark Green	Irregular	Rough	No zonation
27	AB-27	Greenish	Irregular	Rough	No zonation
28	AB-28	Blackish	Irregular	Rough	Zonation
29	AB-29	Whitish	Irregular	Rough	Zonation
30	AB-30	Blackish	Irregular	Rough	Zonation
31	AB-31	Blackish	Irregular	Rough	Zonation
32	AB-32	Whitish	Irregular	Rough	No zonation
33	AB-33	Light Green	Irregular	Rough	Zonation
34	AB-34	Dark Green	Irregular	Rough	Zonation
35	AB-35	Grayish	Irregular	Rough	Zonation
36	AB-36	Whitish	Circular	Rough	No zonation
37	AB-37	Olivaceous black	Irregular	Rough	Zonation
38	AB-38	Light Green	Irregular	Rough	No zonation
39	AB-39	Light green	Irregular	Rough	Zonation
40	AB-40	Blackish	Irregular	Rough	Zonation

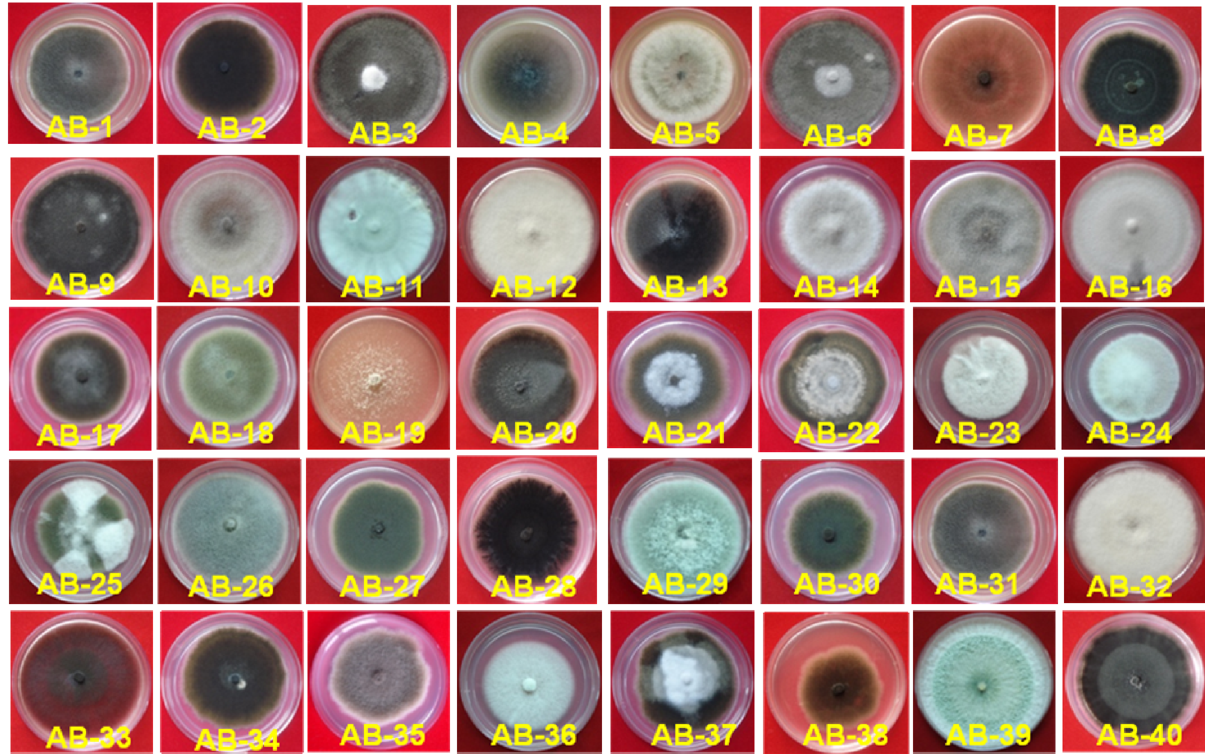


Fig. 4.2.2 Growth characters of *Alternaria brassicae* isolates on Potato Dextrose Agar (PDA) medium

Table 4.2.4 Conidial characters of *Alternaria brassicae* isolates

Sl. No.	Isolates Code	Length (µm)	Width (µm)	Horizontal septa (µm)	Vertical septa (µm)	Sporulation potential ($x10^4$/ml)
1	AB-1	41.3(31-60)	8.3 (6-21)	6.1(5-9)	0.4 (3-0)	1.69
2	AB-2	37.9(36-62)	8.1 (7-18)	6.2(5-10)	0.6 (2-0)	2.01
3	AB-3	38.2(36-57)	6.5 (5-12)	6.6 (6-9)	0.8(2-0)	1.39
4	AB-4	46.9 (39-65)	7.7 (8-15)	7.4 (7-9)	0.6(3-1)	1.81
5	AB-5	61.2 (60-77)	9.5 (7-20)	5.9 (5-8)	1.1(2-1)	1.80
6	AB-6	63.9 (49-87)	10.5 (9-22)	7.1 (7-8)	1.2 (3-1)	1.22
7	AB-7	53.0 (40-82)	7.8 (6-18)	4.8 (4-9)	0.2(3-0)	2.61
8	AB-8	41.9 (33-75)	6.1 (5-17)	4.4 (3-9)	0.6 (3-0)	0.89
9	AB-9	63.5 (49-70)	14.8 (11-20)	7.6 (6-8)	0.8 (2-0)	0.72
10	AB-10	73.8 (38-82)	12.7 (10-21)	7.2 (7-9)	0.6 (2-0)	1.54
11	AB-11	71.3 (54-81)	9.5 (8-18)	8.2 (6-12)	0.2 (3-0)	1.62
12	AB-12	50.2 (41-80)	7.9 (8-16)	5.8 (4-8)	0.4 (2-0)	1.92
13	AB-13	42.9 (33-89)	6.6 (5-15)	6.0(5-9)	0.2 (3-0)	1.32
14	AB-14	43.7 (36-58)	9.7 (7-21)	6.1 (5-11)	0.2 (3-0)	1.49
15	AB-15	39.2 (29-75)	7.9(7-21)	5.0 (4-9)	0.2 (2-0)	2.06
16	AB-16	44.2(31-86)	7.5(6-22)	4.8(3-9)	1.2 (3-1)	2.26
17	AB-17	83.9 (79-87)	12.6(7-18)	6.2 (4-11)	0.6 (2-0)	2.06
18	AB-18	50.8 (35-72)	9.6(6-15)	7.4 (5-9)	0.8 (3-0)	3.90
19	AB-19	53.9 (29-75)	9.9(6-21)	4.0 (3-12)	0.8 (2-0)	1.85
20	AB-20	45.5 (31-60)	8.2(6-11)	6.2 (5-10)	1.2 (3-1)	1.83
21	AB-21	48.0 (37-82)	8.1(7-19)	5.1(5-9)	1.2 (3-0)	2.86
22	AB-22	71.5 (44-81)	10.5(8-16)	6.9(5-11)	0.6 (3-0)	2.86
23	AB-23	34.2 (31-70)	6.2(6-9)	4.0 (3-11)	0.4(3-0)	2.30
24	AB-24	37.9 (33-49)	9.2(6-21)	7.4 (7-12)	1.2(3-0)	2.24
25	AB-25	41.3 (36-68)	7.2(5-17)	6.2 (4-8)	1.4(3-0)	1.53
26	AB-26	89.2 (89-95)	9.2(7-19)	6.1 (4-10)	0.2(3-0)	1.64
27	AB-27	51.5 (43-77)	7.9(6-16)	5.6 (6-10)	1.2(3-1)	3.58
28	AB-28	53.9 (49-57)	8.7(7-21)	7.1 (5-11)	0.6 (3-0)	1.33
29	AB-29	73.8 (65-82)	12.6(6-22)	6.6 (4-10)	0.8 (2-0)	2.30
30	AB-30	40.9 (31-45)	8.9(7-20)	7.6(6-11)	0.6 (3-0)	3.33
31	AB-31	65.5 (51-70)	9.7(9-16)	7.3 (6-12)	0.2(3-0)	1.66
32	AB-32	54.0 (47-72)	11.0(7-21)	6.8 (5-11)	0.8 (2-0)	1.36
33	AB-33	61.5 (54-81)	9.8(6-19)	6.0(4-9)	0.4 (2-0)	2.21
34	AB-34	44.2 (41-50)	8.3(8-21)	5.1 (4-11)	1.6 (2-1)	2.1
35	AB-35	47.9 (43-59)	7.0(6-16)	5.0 (4-8)	1.8(2-1)	1.62
36	AB-36	70.3 (36-78)	12.0(7-21)	5.7(5-9)	0.2(3-0)	1.48
37	AB-37	51.8(30-59)	10.5(7-21)	6.3(5-13)	1.6 (2-1)	2.86
38	AB-38	99.2(62-110)	9.2(8-15)	5.8 (5-8)	1.8 (2-0)	1.90
39	AB-39	74.0 (47-82)	8.6(7-14)	6.2 (6-11)	1.8 (3-1)	2.37
40	AB-40	48.5 (44-81)	8.2(6-18)	5.2 (4-9)	1.2 (2-0)	1.96
CD(0.05)		**8.65**	**2.16**	**0.85**	**0.25**	**0.57**

AB-34, AB-35 and AB-40, while second group (>50 µm) comprises of AB-5, AB-6, AB-7, AB-9, AB-10, AB-11, AB-12, AB-17, AB-18, AB-19, AB-22, AB-26, AB-27, AB-28, AB-29, AB-31, AB-32, AB-33, AB-36, AB-37, AB-38 and AB-39 isolates. Horizontal septa differ significantly between isolates but not the vertical septation. Number of horizontal and vertical septa ranged between 4.0-8.2 and 0.2 -1.8, respectively. Based on the horizontal septa the isolates were categorized into two groups that are < 6 and > 6 septa. The following isolates having < 6 septa AB-5, AB-7, AB-8, AB-12, AB-15, AB-16, AB-19, AB-21, AB-27, AB-34, AB-35, AB-36, AB-38 and AB-40 and remaining isolates having > 6 septa.

The reaction of different isolates on set of fifteen host differentials revealed that all the differentials were susceptible to different isolates of *A. brassicae.* On differentials each isolate behaved differently (Table 4.2.5 and 4.2.6). The per cent disease index varied from 0-100%. The zero per cent indicated highly resistance, 100 per cent indicated highly susceptible reaction. The data revealed that isolate AB-12 could infect all the fifteen host differentials. Uttar Pradesh isolate AB-5 could infect only mustard varieties and one cauliflower variety (Pusa Sharad) and it is not showing any susceptible reaction in other cauliflower and cabbage varieties. The west Bengal isolate AB-19 infected the entire differential varieties except Pusa Mukta (Cabbage). The isolates like AB-9, AB-15, AB-21, AB-29, AB-31, AB-34 and AB-37 infected thirteen varieties among fifteen differentials. Whereas other isolates are not infecting all the fifteen differentials. Among the three crops cauliflower varieties showed wide variation in terms of PDI%. The cauliflower variety Pusa Snow Ball showed 21 resistant reactions and least resistance reaction observed in DC 23,000 variety. In case of Cabbage highest resistant reaction seen in Golden Acre and least in 572 variety. In mustard, highest resistant reaction shown by Pusa Bahar and least resistant reaction was seen in Pusa Bold. In an average cabbage varieties showed more resistant reactions and mustard varieties showed least resistant reaction.

Discussion;

Alternaria brassicae isolates differ greatly in respect to cultural, morphological and pathogenic behaviour. Several workers (Goyal *et al*., 2011, Singh *et al.,* 2007; and Ramegowda and Naik, (2008) have reported cultural, morphological and pathogenic variability among isolates of *Alternaria* spp. In the present investigation cultural, morphological and pathogenic diversity in various isolates of *A. brassicae* infecting crucifers was observed. The results showed that isolates of *A. brassicae* differ in their colony characters, colony diameter and sporulation.

Table 4.2.5. Cross infectivity of *Alternaria brassicae* isolates with different genotypes of cauliflower, cabbage and mustard

Sl. No.	Isolates Code	Cauliflower					Cabbage					Mustard				
		Pusa Sarad	Pusa SnowBall	DC23000	Pusa Megna	Pusa Deepali	Golden Acre	Pusa Mukta	KGMR1	572	Pusa Ageti	Pusa Bold	Pusa Jagannath	Varuna	Pusa Jaikisan	Pusa Bahar
1	AB-1	22.2	0	38.9	0	38.9	0	4.9	0	22.2	0	100	54.3	43.2	39.5	88.9
2	AB-2	6.1	0	22.2	18.5	18.5	0	0	7.4	4.9	0	69.1	88.9	18.5	34.6	12.3
3	AB-3	14.8	0	22.2	7.4	1.2	0	1.9	7.4	17.3	0	25.9	77.8	100	100	83.3
4	AB-4	29.6	0	38.9	60.5	27.8	0	0	0	11.1	0	51.8	43.2	38.8	47.5	100
5	AB-5	6.1	0	0	0	0	0	0	0	0	0	18.5	0	3.7	12.3	12.3
6	AB-6	5.5	0	38.9	3.7	9.9	0	0	0	0	12.3	3.7	4.9	1.2	3.7	3.7
7	AB-7	94.4	50	38.9	64.2	64.2	18.5	7.4	30.2	4.9	14.8	33.9	43.2	0	0	0
8	AB-8	7.4	0	59.3	22.2	12.3	44.4	0	21.6	12.3	14.8	83.9	7.4	64.2	88.9	12.3
9	AB-9	79	0	18.5	40.1	43.2	12.3	4.9	7.4	0	14.8	7.4	15.4	59.3	51.9	59.3
10	AB-10	0	0	0	0	38.9	1.2	1.2	7.4	14.8	55.6	4.9	43.2	47.5	54.3	44.4
11	AB-11	12.3	49.3	24.7	7.4	2.5	7.4	0	0	0	0	56.2	9.8	44.4	22.2	0
12	AB-12	34.5	1.2	38.9	18.5	9.9	7.4	18.5	12.3	7.4	4.9	3.7	9.8	18.5	12.3	5.55
13	AB-13	25.9	0	30.2	56.2	29.6	5.5	1.2	1.2	77.8	7.4	0	74.1	5.5	0	0
14	AB-14	0	0	59.3	43.2	0	0	9.3	77.8	44.4	2.5	24.7	29.6	0	0	0
15	AB-15	69.1	3.7	59.3	40.7	18.5	0	66.7	34.6	66.7	0	12.3	21.6	7.4	12.3	43.2
16	AB-16	0	4.9	18.5	27.8	7.4	0	14.8	0	25.9	0	64.2	18.5	34.5	39.5	34.5
17	AB-17	14.8	0	29.6	0	18.5	12.3	39.5	0	7.4	0	34.6	15.4	51.8	61.1	56.2
18	AB-18	0	0	0	1.85	0	18.5	0	7.4	12.3	12.3	38.9	0	94.4	12.3	34.2

19	AB-19	47.5	9.2	18.5	37	18.5	3.7	0	17.3	21.6	11.1	1.2	7.4	34.5	29.6	64.2
20	AB-20	4.9	0	2.5	18.5	56.2	1.2	1.2	25.9	0	18.5	56.2	9.8	56.2	0	9.8
21	AB-21	0.61	12.3	22.2	40.7	3.7	2.5	14.8	0	0	44.4	12.3	51.8	33.3	12.3	1.2
22	AB-22	30.8	1.8	9.2	0	0.6	0	0	9.2	9.2	30.2	12.3	0	0	25.3	51.8
23	AB-23	0	0	34.6	100	51.8	1.2	0	6.2	0	0	1.2	0	0	18.5	12.3
24	AB-24	69.1	34.5	43.2	44.4	44.4	0	0	1.8	0	0	2.5	15.4	12.3	94.4	12.3
25	AB-25	0	0	59.3	88.9	100	0	14.8	9.2	44.4	9.2	94.4	51.8	8.6	88.9	1.2
26	AB-26	69.1	94.4	100	0	25.9	0	54.3	0	11.1	9.2	0	15.4	15.4	0	4.9
27	AB-27	94.4	69.1	69.1	100	18.5	9.9	66.7	0	0	0	12.3	40.7	40.7	40.7	0
28	AB-28	59.2	25.9	54.3	43.2	18.5	0	0	7.4	12.3	21.6	0	18.5	15.4	88.9	18.5
29	AB-29	64.1	0.61	64.2	51.9	18.5	0	0	29.6	3.7	34.6	4.9	64.8	74.1	100	64.8
30	AB-30	0	47.5	38.9	100	12.3	3.7	59.3	24.7	0	0	12.3	15.4	15.4	15.4	15.4
31	AB-31	1.2	0	18.5	38.9	0	3.7	43.2	4.9	7.4	7.4	34.6	21.6	30.9	54.3	21.6
32	AB-32	64.1	0	47.5	43.2	25.9	0	0	4.9	14.8	9.2	0	43.2	3.7	54.3	47.5
33	AB-33	47.5	44.4	34.6	33.3	12.3	1.2	0	0	56.2	6.2	12.3	4.9	0	15.4	1.2
34	AB-34	24.6	66.6	40.7	1.2	2.4	0	12.3	0	38.9	1.9	51.8	1.2	18.5	18.5	9.2
35	AB-35	34.5	0	69.1	1.2	1.2	7.4	7.4	0	15.4	1.2	2.5	1.2	44.4	34.5	0
36	AB-36	0	0	100	100	3.7	0	0	0	0	50	2.5	7.4	49.4	30.2	12.3
37	AB-37	100	66.6	38.9	100	83.3	69.1	7.4	1.2	34.6	25.8	1.2	1.2	0	0	1.8
38	AB-38	14.8	34.9	29.6	0	59.3	3.7	1.2	7.4	69.1	0	43.2	0	2.5	0	0
39	AB-39	0	66.9	43.2	17.3	34.6	7.4	4.9	7.4	0	0	51.8	0	1.2	12.3	0
40	AB-40	12.3	0	47.5	3.7	0	1.9	0	21.6	0	14.9	4.9	15.4	74.1	83.3	47.5

Table 4.2.6. Cross infectivity reaction of *Alternaria brassicae* isolates with different genotypes of cauliflower, cabbage and mustard

Sl. No.	Isolates Code	Cauliflower					Cabbage					Mustard				
		Pusa Sarad	Pusa SnowBall	DC23000	Pusa Megna	Pusa Deepali	Golden Acre	Pusa Mukta	KGMR1	572	Pusa Ageti	Pusa Bold	Pusa Jagannath	Varuna	Pusa Jaikisan	Pusa Bahar
1	AB-1	R	HR	MR	HR	MR	HR	R	HR	R	HR	S	MS	MR	MR	S
2	AB-2	R	HR	R	R	R	HR	HR	R	R	HR	MS	S	R	MR	R
3	AB-3	R	HR	R	R	R	HR	R	R	R	HR	MR	S	S	S	S
4	AB-4	MR	HR	MR	MS	MR	HR	HR	HR	R	HR	MS	MR	MR	MR	S
5	AB-5	HR	HR	HR	HR	HR	HR	HR	HR	HR	HR	R	HR	R	R	R
6	AB-6	R	HR	MR	R	R	HR	HR	HR	HR	R	R	R	R	R	R
7	AB-7	S	HR	MR	MS	MS	R	R	MR	R	R	MR	MR	HR	HR	HR
8	AB-8	R	HR	MS	R	R	MR	HR	R	R	R	S	R	MS	S	R
9	AB-9	S	HR	R	MR	MR	R	R	R	HR	R	R	R	MS	MS	MS
10	AB-10	HR	HR	HR	HR	MR	R	R	R	R	MS	R	MR	MR	MS	MR
11	AB-11	R	MR	R	R	R	R	HR	HR	HR	HR	MS	R	MR	R	HR
12	AB-12	MR	R	MR	R	R	R	R	R	R	R	R	R	R	R	R
13	AB-13	MR	HR	MR	MS	MR	R	R	R	S	R	HR	S	R	HR	HR
14	AB-14	HR	HR	MS	MR	HR	HR	R	S	MR	R	R	MR	HR	HR	HR
15	AB-15	MS	R	MS	MR	R	HR	MS	MR	MS	HR	R	R	R	R	MR
16	AB-16	HR	R	R	MR	R	HR	R	HR	MR	HR	MS	R	MR	MR	MR
17	AB-17	R	HR	MR	HR	R	R	MR	HR	R	HR	MR	R	MS	MS	MS
18	AB-18	HR	HR	HR	R	HR	R	HR	R	R	R	MR	HR	S	R	MR
19	AB-19	MR	R	R	MR	R	R	HR	R	R	R	R	R	MR	MR	MS
20	AB-20	R	HR	R	R	MS	R	R	MR	HR	R	MS	R	MS	HR	R
21	AB-21	HR	R	R	MR	R	R	R	HR	HR	MR	R	MS	MR	R	R
22	AB-22	MR	R	R	HR	HR	HR	HR	R	R	MR	R	HR	HR	MR	MS
23	AB-23	HR	HR	MR	S	MS	R	HR	R	HR	HR	R	HR	HR	R	R
24	AB-24	MS	MR	MR	MR	MR	HR	HR	R	HR	HR	R	R	R	S	R

Sl. No.	Isolates Code	Cauliflower					Cabbage					Mustard				
		Pusa Sarad	**Pusa SnowBall**	**DC23000**	**Pusa Megna**	**Pusa Deepali**	**Golden Acre**	**Pusa Mukta**	**KGMR1**	**572**	**Pusa Ageti**	**Pusa Bold**	**Pusa Jagannath**	**Varuna**	**Pusa Jaikisan**	**Pusa Bahar**
25	AB-25	HR	HR	MS	S	S	HR	R	R	MR	R	S	MS	R	S	R
26	AB-26	MS	S	S	HR	MR	HR	MS	HR	R	R	HR	R	R	HR	R
27	AB-27	S	MS	MS	S	R	R	MS	HR	HR	HR	R	MR	MR	MR	HR
28	AB-28	MS	MR	MS	MR	R	HR	HR	R	R	R	HR	R	R	S	R
29	AB-29	MS	HR	MS	MS	R	HR	HR	MR	R	MR	R	MS	MS	S	MS
30	AB-30	HR	MR	MR	S	R	R	MS	R	HR	HR	R	R	R	R	R
31	AB-31	R	HR	R	MR	HR	R	MR	R	R	R	MR	R	MR	MS	R
32	AB-32	MS	HR	MR	MR	MR	HR	HR	R	HR	R	HR	MR	R	MS	MR
33	AB-33	MR	MR	MR	MR	R	R	HR	HR	MS	R	R	R	HR	R	R
34	AB-34	R	MS	MR	R	R	HR	R	HR	MR	R	MS	R	R	R	R
35	AB-35	MR	HR	MS	R	R	R	R	HR	R	R	R	R	MR	MR	HR
36	AB-36	HR	HR	S	S	R	HR	HR	HR	HR	MS	R	R	MR	MR	R
37	AB-37	S	MS	MR	S	S	MS	R	R	MR	MR	R	R	HR	HR	R
38	AB-38	R	MR	MR	HR	MS	R	R	R	MS	HR	MR	HR	R	HR	HR
39	AB-39	HR	MS	MR	R	MR	R	R	R	HR	HR	MS	HR	R	R	HR
40	AB-40	R	HR	MR	R	HR	R	HR	R	HR	R	R	R	MS	S	MR

Most of the isolates of *A. brassicae* showed different mycelia pigmentation on PDA, CDA and CLEA. The colour varied from whitish to black. Ramegowda and Naik, (2008) also noticed different pigmentation on isolates of *Alternaria* spp. Other colony characters such as texture, culture margin and appearance of mycelial growth were highly varied among the isolates. The culture characters also revealed the difference in zonation pattern of the isolates on PDA, CDA and CLEA. This type of variations in zonation may be due to the lack of essential nutrition for growth of *A. brassicae* isolates on PDA and CDA. Environmental causes such pH, temperature, relative humidity; quantity of medium might have influenced the colour, zonation, margin shape and sporulation pattern of different isolates (Humpherson-Jones and Phelps, 1989).

The spore production of forty isolates on PDA media mentioned above showed variation in sporulation pattern. The isolate AB-18 produced higher (3.90×10^4 / ml) number of spores than other isolates. Most of the isolates depicted high growth rate and high number of spore production on CLEA followed by PDA and CDA. Umamaheshwari *et al.*, 2008, also observed same pattern in *Alternaria alternata* and *A. cumcummerina* on
Watermelon Leaf Extract media. Colony diameter, colony colour, texture, spore morphology, sporulation pattern on different medium showed high variation among forty isolates tested. These variations were not according to the geographical location and plants (Cabbage, cauliflower and mustard). Earlier authors also reported the variability in *Alternaria* spp. associated with different crop plants showing non lineage with samples collected from different geographical regions.

All the isolates were found pathogenic in nature against their respective host. The reaction of different isolates on set of fifteen differential varieties revealed that all the differentials were susceptible to different isolates of *A. brassicae* on differentials each isolate behaved differently. The finding of the present study clearly indicates the existence of pathogenic variability in *A. brassicae.* Awasthi and Kolte (1989) distinguished three isolates A, C and D from Pant Nagar on the basis of pathogenesis on crucifers. Mehta *et al.,* 1997 had also indicated that different isolates collected from different agro-climatic zones of India behaved differently on the set of seventeen host differentials. Wide variation in virulence among the races of *A. brassicae* have also been observed by Saharan and Kadian (1983) though studies were based on isolates collected from different species of rapeseed and mustard from same location. In

the present investigation the isolates were collected from different agro-climatic regions of India and gave different reactions which strongly suggest existence of variability in *A. brassicae*.

4.3. RESEARCH PAPER-III

Biochemical and molecular characterization of *Alternaria brassicae* isolates

Abstract

Alternaria brassicae is the most important pathogen on all cruciferous crops and cause adverse effect on both quality and quantity. Present investigation was carried out to know the biochemical and molecular variability in *A. brassicae* causing black leaf spot. Forty isolates of *A. brassicae* were collected from different agro climatic regions of India. Biochemical and molecular characterization was done on the forty isolates. All the isolates showed high level of variability *in vitro* in respect to enzyme production, protein profile, Randomly Amplified Polymorphic DNA (RAPD) markers and Internal Transcribed Spacer (ITS) region sequences. Most of the isolates produced more cellulase than lipase and pectinase. The pectinase production also has the correlation with disease development of the particular isolate. Based on the protein profiling the isolates were grouped into three groups. Forty random primers were screened for variability studies and total of 257 bands were obtained out of which 204 bands (80.3 per cent) were polymorphic. The variability of the isolates were analysed for the RAPD marker and isolates were grouped into six distinct clusters of 0.67 similarity coefficient. Some isolates from Maharashtra, Uttar Pradesh, and Rajasthan were very similar and occurred in a single cluster. Among the two techniques used to identify the polymorphism within *A. brassicae* isolates revealed that RAPD marker given more polymorphism than the ITS region.

Key words:

A. brassicae; Black leaf spot; Cellulase; Lipase; Pectinase; RAPD; ITS

Introduction

Alternaria brassicae causes black spot in crucifers, which can cause considerable yield reduction in *Brassica* spp. (Sigareva and Earle, 1999a&b). It is causing up to 47 per cent yield losses with no proven source of resistance against the disease reported till date in any of the hosts (Meena *et al.,* 2012). *Alternaria* species are present worldwide and attack a large number of brassicaceous crops. Among them the common hosts of economic importance are rapeseed-

mustard, cabbage, cauliflower, broccoli, turnip, radish and brussels sprouts. *A. brassicae* can affect host species at all stages of growth including seeds. On seedlings symptoms include dark stem lesions immediately after germination that results in damping-off or stunted seedlings. When older plants become infected, Alternaria symptoms often occur on the older leaves, since they are closer to the soil and are more readily infected as a consequence of rain splash or windblown rain. Late infection or infection of older leaves does not characteristically reduce yields and can be controlled through intensive removal of infected leaves. Fruit-bearing branches and seed pods show dark or blackened spots that result in yield loss due to premature pod ripening and shedding of the seeds. Infection can also occur on the fruit, before or after harvest. A common symptom of broccoli and cauliflower infection is a browning that occurs on the head. The pathogen enters the host either through injury/natural opening or through producing haustoria. During pathogenesis, pathogen has to overcome the barrier of cell wall and to overcome this barrier; the enzymes capable of degrading the cell wall are induced by the pathogen. Among the different enzyme cellulase, pectinase, lipase and brassinin hydrolase plays an important role (Jain and Dhawan, 2008). Also severity of Alternaria blight on Brassicas differs among seasons and regions as also between individual crops within a region. This may be due to existence of variability among isolates of *Alternaria* spp. Alternaria is an extremely diverse genus of the phylum Ascomycota consisting of both saprophytic and pathogenic species. The taxonomy of *Alternaria* species is based predominantly on spore morphology, cultural characteristics and host pathogen association. Sometimes protein based markers like SDS-PAGE also used to distinguish the microorganism in races level (Karthikeyan and Thajjudin, 2010). Recent advances in fungal taxonomy and identification and differentiation of *Alternaria* species have been made through DNA based assays such as RFLP, RAPD, AFLP, rep-PCR and species-specific PCR. RAPD analysis allows quick assessment of genetic variability of various taxa and has been used to study inter and intra specific variation of any fungal isolates (Pryor and Michailides, 2002; Gherbawy, 2005 and Mercado *et al*., 2006).This analysis proved to be an efficient method for checking the genetic variability of *A. cassia* and *A. alternata* isolates occurring in *Senna optusifolia* and also for distinguishing *Alternaria* sp. (Tigano *et al*., 2003). RAPD markers are very quick and easy to handle but lack reproducibility (Karp *et al*., 1997; Hansen *et al*., 1998; Jones *et al*., 1997 and Virk *et al*., 2000). In fungi including *Alternaria* species, differences in sequences of ribosomal DNA have been used as a rapid method for

studying the evolution and identification of species (Jasalavich *et al*, 1995; Kusaba and Tsuge, 1995 and Morales *et al*., 1995).

The purpose of this study was to examine biochemical and molecular diversity among *Alternaria brassicae* isolates from different agro-climatic regions of India, to determine the spatial distribution of this diversity and evaluate the pathogenicity. In addition, a study was carried out to evaluate phylogenetic relationship among *A. brassicae* isolates using a variety of molecular tools as an effort to further understand the diversity status among the isolates.

Material and methods

Collection, identification and maintenance of isolates

Isolation and purification of *Alternaria brassicae* cultures was done from disease infected leaves of mustard, cabbage and cauliflower were collected from different agro-climatic regions of India. *A. brassicae* cultures were identified based on morphological characteristics. The cultures were purified by single spore isolation method and stored on PDA slants at 4° C for further study.

Biochemical characterization;

Enzyme assay

For the assay of enzyme activity the isolates *Alternaria brassicae* were grown on minimal synthetic medium (MSM). The qualitative assay was performed before the quantitative assay, the qualitative assay performed in the petri plates. Minimal synthetic media was used for the entire enzyme assay studies. In the minimal synthetic media *Alternaria brassicae* was inoculated and incubated at 29°C in an inverted position for 2-5 days. After the visible growth seen the plates were flooded with 1% aqueous solution at hexadecyltrimethyl ammonium bromide/congo red. The plates were kept in shaker for 4 hr. Then the aqueous solution were drained out and poured with 1% NaCl and incubated for another 1 hr and drained out. A clear area around the colony of *A. brassicae* indicates production of respective enzymes. The medium was supplemented with respective carbon source (Cellulose for cellulase, pectin for pectin and olive oil for lipase).

Quantitative assay of cellulase, lipase and pectinase was performed by spectro-photometric method. Cellulase activity was assayed by measuring the release of reducing sugar with DNS (Miller, 1959). The assay mixture contained 1 ml of 0.5 per cent cellulose (Sigma Co.) suspended in 50Mm citrate phosphate buffer (pH 4.8) and 1 ml of culture filtrates of different

Alternaria Brassicae strains in 15 ml test tubes. The reaction mixture was incubated for 30 min at 50^0 C and then centrifuged at 12000 rpm for 15 min at 4^0 C. The reaction was arrested by adding 3 ml of 1% DNS (dinitrosalicylate) reagent in 1 M NaOH and followed by heating for 10 min at 100^0 C to develop the red-brown colour. While it was hot 1ml of 40% Rochelle salt (potassium sodium tartrate) was added to stabilize the color. The blanks were made in the same way using distilled water bath, the absorbance was measured with a spectrophotometer (Systronics spectrophotometer) at 540nm and the glucose content was obtained by comparing with glucose standard prepared by the same procedure.

Quantitative determination of Lipase activity was performed by measuring the increase in absorbance at 410nm in a visible spectrophotometer caused by the release of *p*-nitrophenol after hydrolysis of *p*-nitrophenyl palmitate (*p*NPP) as the substrate at 37^0C for 30 minutes (modified from the method described by Winkler and Stuckmann, 1979). A 30 mg of pNPP dissolved in 10 ml isopropanal was emulsified in 90ml of 50mM Tris-HCl buffer pH 7.0, containing 1.8% (v/v) Triton X-100 and 100mg of gum Arabic. To initialize the reaction, a 0.1ml of enzyme solution was mixed with 0.9ml of pNPP reaction mixture and incubated at 37^0C for 30 minutes. The reaction was stopped by the addition of 1ml of 1M Na_2CO_3. The absorbance was recorded at 410nm. One unit (U) was defined as the amount of enzyme that liberated 1 µmol *p*-nitrophenol per min, in the assay conditions.

Pectinase activity was assayed by the colorimetric method of Miller. Briefly, 0.5 ml of cell free supernatant was incubated with 0.5 ml of pectin in 0.1 M acetate buffer with pH 6.0 and the reaction mixture was incubated at 40°C for 10 minutes in static condition. After adding 1 ml of DNS reagent, the mixture was boiled for 5 min at 90°C. The reaction was stopped by adding 1ml of Rochelle's salt. Then the mixture was diluted by adding 2 ml of de-ionized water. The absorbance was measured Spectrophotometrically at 595 nm. A standard graph was generated using standard glucose solution.

One unit of enzyme activity was defined as the amount of enzyme in one 1 ml of the reaction mixture that released 1µmol of reducing sugar in case of cellulase and pectinase and *p*-nitrophenol in case of lipase under the assay condition.

Brassinin assay

The anti-fungal activity of brassinin to and *A. brassicae* (isolate AB-16) was investigated using the following assay.

Spore germination inhibition assay

DMSO (control) or a DMSO solution of brassinin (final concentration 0.12–0.50 mM) was added to spores suspensions of *A. brassicae* isolates ($1.8X10^5$ spores ml_1) in minimal medium containing 1% Tween 80 and incubated on a shaker at 25±2°C. The germinated and ungerminated spores were counted in ten random fields of each well with an inverted microscope at 40X magnification.

SDS-PAGE analysis

Identification of variations in *Alternaria brassicae* was done using Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The fungal protein was extracted from different isolates. In protein isolation the mycelial mats were ground in pre-chilled sterilized pestle and mortar into fine powder with liquid nitrogen and transferred to centrifuge tubes, containing extraction buffer (Tris-HCl 0.05 M pH 7.4, Glycerol 5%, SDS 0.5% and β-Mercaptoehanol 0.1%). The tubes were allowed to stand for one hour on ice. The samples were then centrifuged at 10,000 rpm for 45 min. at 4^oC, the clear supernatant was collected. To these seven volumes chilled acetone was added and kept at -20^oC for overnight. The proteins were precipitated by centrifugation at 10000 rpm for 20 min at 4^oC, the pellet was washed two times with cold acetone and air dried. The pellet was dissolved in sample buffer for SDS-PAGE and samples were loaded after denaturation. Samples were loaded and electrophoresed at 80 Volts till the sample is in stacking gel and voltage raised up to 120 volts for separating gel. After the electrophoresis, the gel was stained with Coomassive brilliant blue for overnight and destained. The SDS-PAGE Gels were scored on the basis of the presence (1) or absence (0) of each band for all isolates.

Molecular variability

Fungal DNA extraction and purification

DNA was extracted by CTAB protocol (Murray and Thompson, 1980). The mycelium was grown for seven days on PDB and harvested, dried, powdered with liquid nitrogen. The powder was transferred into a centrifuge tube and 10 ml of CTAB (DNA extraction buffer) was added. This was incubated at 65ºC in a water bath for 30 min with intermittent shaking. Equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added with 2–3 min slow inversion and centrifuged at 13,000 rpm for 15 min at 4ºC. The aqueous layer was taken in a fresh tube and 0.8 volume of ice cold isopropanol was added and incubated at -20ºC for 30 min.

After incubation, it was again centrifuged at 13,000 rpm for 15 min at 4ºC. The supernatant was discarded and pellet was washed with 70 per cent ethanol. The pellet was dried and dissolved in 200 µl of 1X TE buffer.

RNA in the total nucleic acid extracted was removed by RNAse treatment. The reaction mixture composition was as follows: Total nucleic acid ~ 200 ng; RNAse- 2 µl, sterile distilled water 50 µl. The reaction mixture was incubated at 37°C for one hr. DNA concentration of samples and purity was determined by taking ultraviolet absorbance at 260 nm and 280 nm on spectrophotometer (NanoDrop, USA) followed by agarose gel electrophoresis.

Random primers and PCR amplification

Different PCR reactions were tested for obtaining best amplification (Cobb and Clarkson, 1994). DNA of different isolates was amplified by RAPD method (Williams *et al*., 1990) using 31 decamer arbitrary oligonucleotide primers (Operon Technology, USA). The PCR-amplification reaction was optimized with various concentrations of $MgCl_{2,}$ dNTPs, primer and template DNA. The reaction was performed in 15µl volume containing DNA template 50 ng, *Taq* DNA polymerase 1 U, primer (15 µM), (10-mer primer), $MgCl_2$ (50 µM), dNTPs mix 50 µM and 10X PCR buffer. Nuclease free water was used to bring the reaction volume to 15µl. The DNA amplification was performed by using Mastercycler^{TM} gradient thermal cycler (Bio Rad, Germany). The PCR conditions were consisted of denaturation of 5 min at 94°C followed by 32 cycles of 45 sec at 93°C, annealing for 30 sec at 34°C, and 50 sec at 72°C with a final extension for 8 min at 72°C. Amplified fragment were resolved in 1.3 per cent agarose gels in 1x TAE electrophoresis buffer pre-stained with ethidium bromide along with 1kb DNA ladder (Fermentas Life Sciences, Canada) and imaged with digital gel documentation system (Bio Rad Gel Doc system).

ITS Primers and PCR amplification

The ITS regions and the 5.8S rDNA of 21 isolates (AB-9, AB-10, AB-11, AB-17, AB-22, AB-23, AB-24, AB-25, AB-26, AB-27, AB-28, AB-29, AB-30, AB-31, AB-32, AB-33, AB-34, AB-35, AB-36, AB-38 and AB-39) of the pathogen representing various RAPD groups were amplified with a set of primers ITS1 (TCCGTAGGTGAACCTGCGG) and ITS4 (TCCTCCGCTTATTGATATGC) as described by White *et al.* (1990). The PCR-amplification reaction was optimized with various concentrations of $MgCl_{2,}$ dNTPs, primer and template DNA. The cycle parameters included an initial denaturation step at 94°C for 5 min followed by 35

cycles consisting of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min and final extension for 10 min at 72°C. Amplified products were separated on 1.4% agarose gel in 1X TAE buffer, pre-stained with ethidium bromide (1 µg ml^{-1}) and electrophoresis was carried out at 60 volts for 2 hours in TAE buffer. 1 kb ladder (Fermentas Life Sciences, Canada) was used as a marker. The gel was observed under ultraviolet light using Bio Rad Gel Doc system.

ITS sequencing

The ITS amplified bands (~600 bp) of 21 isolates isolates (AB-9, AB-10, AB-11, AB-17, AB-22, AB-23, AB-24, AB-25, AB-26, AB-27, AB-28, AB-29, AB-30, AB-31, AB-32, AB-33, AB-34, AB-35, AB-36, AB-38 and AB-39) were eluted and purified using QIAGEN gel extraction and purification kits (Promega, USA) as per manufacturer's instructions. The eluted and purified DNA samples were sequenced at SciGenome, India.

The nucleotide sequences were subjected to BLAST analysis on World Wide Web (http://www. ncbi.nih.gov/index.html) for homology searching. Sequences were then submitted to GenBank at National Center for Biotechnology Information (NCBI) and accessions were obtained. The multiple sequence alignment and pair wise alignment were made using BioEdit version 7.0.5 (Hall, 1999). Phylogeny tree was constructed based on the maximum nucleotide sequence similarity by using ClustalW 1.8 sequence alignment selecting Bootstrap neighboring joint by MEGA 4.1 programme.

Results

Enzyme assay

All the *A. brassicae* isolates were screened for enzyme production in petri plates. Most of the isolates produced clear zone around the colony in minimal synthetic media (Table 4.3.1; Fig. 4.3.1). The slow growing isolates like AB-1 and AB-37 shown negative result (no clear zone) in cellulase assay. In pectinase assay also AB-1 and AB-37 shown negative result. In case of lipase assay more number of isolates shown negative results with no clear zonation in the isolates *viz.,* AB-1, AB-7, AB-9, AB-12, AB-20, AB-23, AB-26, AB-37 and AB-39.

Quantitative assay enzyme assay of the pathogen was done by spectrophotometric methods (Table 4.3.2; Fig 4.3.2), cellulase producing capacity of the pathogen were varied from 13.743 to 1.133 IU/mg. The highest cellulase production was recorded in AB-22 isolate and lowest was recorded in AB-37 isolate. Lipase production varied from 9.104 to 1.222 IU/mg. The

Table 4.3.1. Qualitative enzyme analysis based on clear zone around the colony in minimal synthetic media.

Sl.No.	Isolates Code	Cellulase	Lipase	Pectinase
1	AB-1	Negative	Negative	Negative
2	AB-2	Positive	Positive	Positive
3	AB-3	Positive	Positive	Positive
4	AB-4	Positive	Positive	Positive
5	AB-5	Positive	Positive	Positive
6	AB-6	Positive	Positive	Positive
7	AB-7	Positive	Negative	Positive
8	AB-8	Positive	Positive	Positive
9	AB-9	Positive	Negative	Positive
10	AB-10	Positive	Positive	Positive
11	AB-11	Positive	Positive	Positive
12	AB-12	Positive	Negative	Positive
13	AB-13	Positive	Positive	Positive
14	AB-14	Positive	Positive	Positive
15	AB-15	Positive	Positive	Positive
16	AB-16	Positive	Positive	Positive
17	AB-17	Positive	Positive	Positive
18	AB-18	Positive	Positive	Positive
19	AB-19	Positive	Positive	Positive
20	AB-20	Positive	Negative	Positive
21	AB-21	Positive	Positive	Positive
22	AB-22	Positive	Positive	Positive
23	AB-23	Positive	Negative	Positive
24	AB-24	Positive	Positive	Positive
25	AB-25	Positive	Positive	Positive
26	AB-26	Positive	Negative	Positive
27	AB-27	Positive	Positive	Positive
28	AB-28	Positive	Positive	Positive
29	AB-29	Positive	Positive	Positive
30	AB-30	Positive	Positive	Positive
31	AB-31	Positive	Positive	Positive
32	AB-32	Positive	Positive	Positive
33	AB-33	Positive	Positive	Positive
34	AB-34	Positive	Positive	Positive
35	AB-35	Positive	Positive	Positive
36	AB-36	Positive	Positive	Positive
37	AB-37	Negative	Negative	Negative
38	AB-38	Positive	Positive	Positive
39	AB-39	Positive	Negative	Positive
40	AB-40	Positive	Positive	Positive

Negative-No clear zone; Positive-Clear zone

Table 4.3.2. Quantitative enzyme analysis based on spectrophotometric method.

Sl. No.	Isolates Code	Cellulase activity (IU/mg)	Lipase activity (IU/mg)	Pectinase activity (IU/mg)
1	AB-1	3.700	5.153	7.400
2	AB-2	8.565	7.244	7.537
3	AB-3	6.018	3.126	4.904
4	AB-4	8.653	1.932	8.355
5	AB-5	7.603	2.187	7.349
6	AB-6	8.363	6.861	7.856
7	AB-7	2.483	6.403	1.987
8	AB-8	7.201	5.632	6.705
9	AB-9	5.946	3.279	5.286
10	AB-10	10.120	3.207	9.487
11	AB-11	9.823	6.712	8.186
12	AB-12	9.009	5.537	7.078
13	AB-13	9.388	3.932	7.731
14	AB-14	5.968	2.361	5.570
15	AB-15	8.114	1.821	6.491
16	AB-16	10.845	5.001	9.569
17	AB-17	9.717	4.479	7.848
18	AB-18	8.538	4.963	6.831
19	AB-19	12.333	9.104	9.737
20	AB-20	6.866	2.469	5.722
21	AB-21	12.515	4.696	9.794
22	AB-22	13.743	3.650	11.981
23	AB-23	7.354	5.058	6.435
24	AB-24	7.551	5.594	6.293
25	AB-25	7.698	2.947	5.463
26	AB-26	12.658	5.392	11.035
27	AB-27	8.868	3.101	7.645
28	AB-28	7.057	2.387	6.485
29	AB-29	7.827	2.661	9.487
30	AB-30	7.848	3.774	7.624
31	AB-31	5.663	1.304	7.551
32	AB-32	5.899	3.298	6.703
33	AB-33	6.271	3.150	5.017
34	AB-34	6.840	3.891	5.597
35	AB-35	6.568	2.929	5.473
36	AB-36	6.167	2.318	5.570
37	AB-37	1.133	1.222	2.643
38	AB-38	6.791	2.931	6.557
39	AB-39	8.409	1.960	10.091
40	AB-40	6.840	4.629	6.218

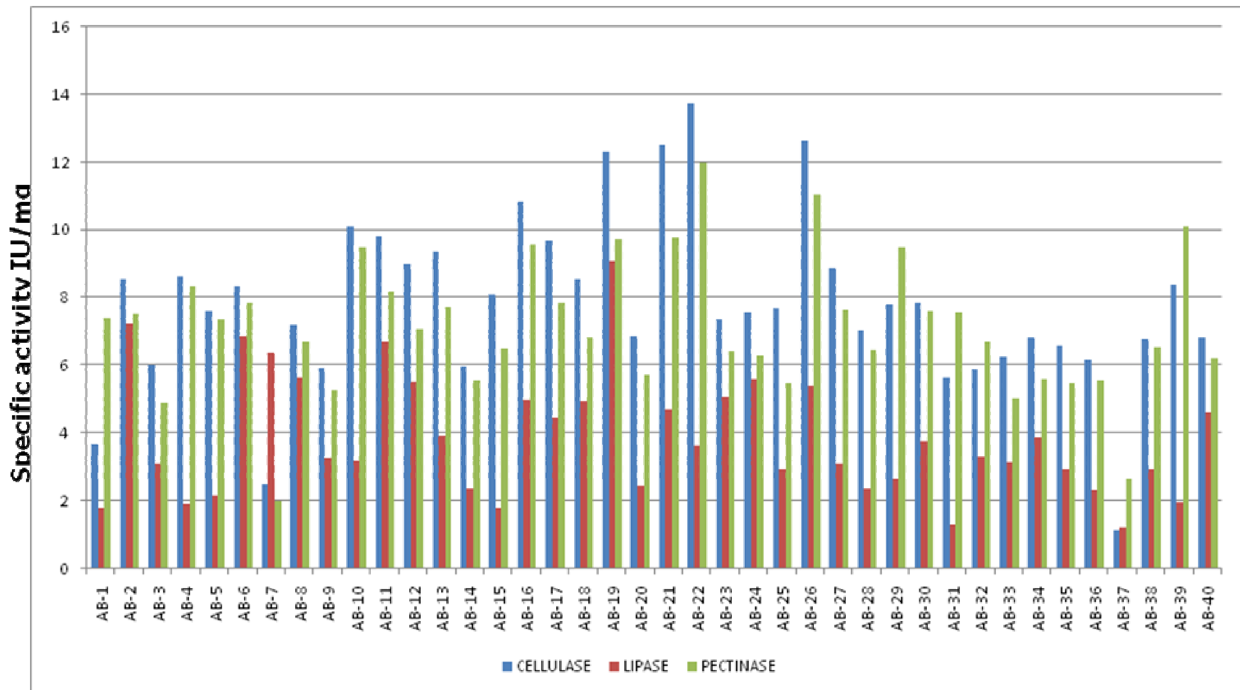


Fig. 4.3.1. Graph showing quantitative enzyme analysis based on spectrophotometric method

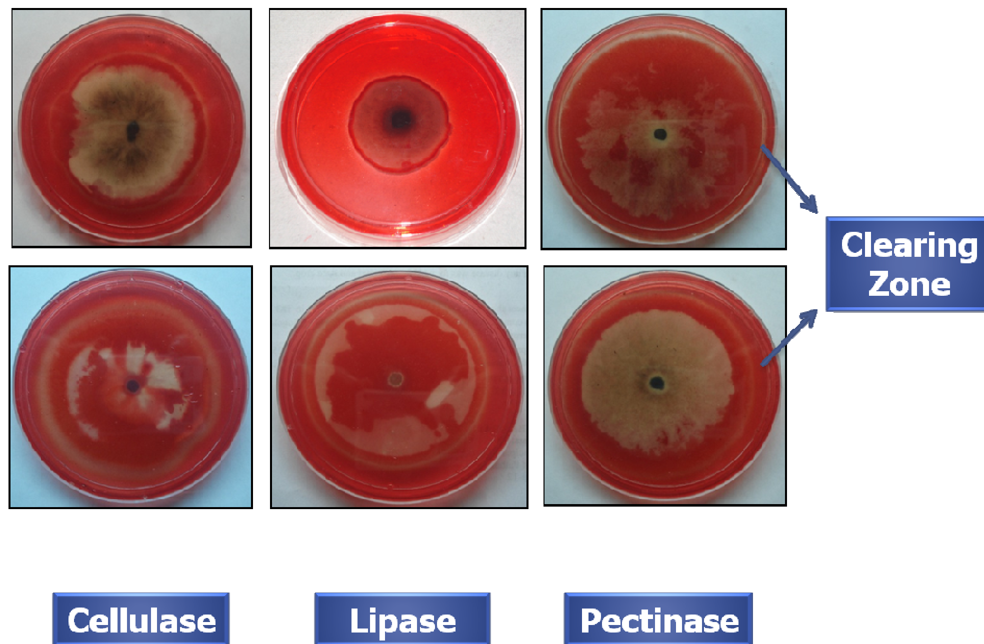


Fig. 4.3.2 Qualitative enzyme assay showing clearance zone in different substrates by *Alternaria brassicae* isolates

highest lipase production was recorded from AB-19 isolate and lowest was recorded from AB-37 isolate. Pectinase production varied from 11.981 to 2.643 IU/mg. The highest pectinase production was recorded in AB-22 isolate and lowest was recorded in AB-37 isolate. The brassinin added in the media drastically reduced the spore germination potential of the pathogen (Table 4.3.3)

Protein Profiling

The total proteins of 40 strains of *Alternaria brassicae* were analyzed, separately by SDS-PAGE. Protein profile representing the relative position and intensities of bands were divided into two zones from higher molecular weight(A) to lower molecular weight(B) and three different band intensities *viz.* dark, medium dark and light bands (Fig. 4.3.3).

Protein Profiling Analysis by SDS-PAGE

Total number of protein bands ranged from 4kD to 28kD spread over into A and B zone with different intensity bands. On the basis of total number of bands and their intensity, strains were classified into three groups (Table 4.3.4). The total soluble protein patterns among them were similar. There was absence of bands at low molecular weight region (approximately 20.1 kD) which is AB4, AB13, AB22, AB27 and AB36, which was characteristic of the protein profiles of all the isolates and there was no bands above 95kD in all the isolates. There were three strains (AB9, AB32 AND AB34) shows 98 per cent monomorphic bands as well as up to 95kD region from lower molecular weight (10kD), around 35kD of band intensity similar in these three strains but AB9 strains showing all the bands showing more intensity. The strain AB17 showed 23 bands whereas strain AB14, AB20, AB24 showed 23 bands but AB17 50 per cent polymorphic with strains AB14, AB20, AB24. The only one strain AB17 has more number of bands are of high intensity and 70 kD, 43 kD and 30 kD of proteins are different from other strains.

RAPD analysis

Molecular variability analysis 40 Random primers were screened out of that 31 RAPD primers given reproducible bands. All 31 RAPD primers screened for amplification of DNA of 40 isolates of *A. brassicae* produced reproducible and scorable bands ranging from 4 to 12 in number with 400-1500 bp molecular size (Fig 4.3.4). A total of 257 bands were obtained out of which 204 bands (80.30 per cent) were polymorphic (Table 4.3.5). Values of the Jaccard's similarity co-efficient ranged from 57 per cent between AB-6 and AB-20 to other isolates, and

Table 4.3.3. Germination percentage of *Alternaria brassicae* in presence of brassinin hydrolase.

Sl. No.	Isolates Code	Spore germination (%)	
		Control	Brassinin Hydrolase treated
1	AB-1	96	34
2	AB-2	89	21
3	AB-3	88	18
4	AB-4	86	22
5	AB-5	86	11
6	AB-6	86	8
7	AB-7	84	13
8	AB-8	88	25
9	AB-9	91	20
10	AB-10	95	12
11	AB-11	97	10
12	AB-12	91	7
13	AB-13	92	14
14	AB-14	96	11
15	AB-15	90	10
16	AB-16	92	13
17	AB-17	90	10
18	AB-18	91	11
19	AB-19	90	10
20	AB-20	93	08
21	AB-21	91	11
22	AB-22	95	10
23	AB-23	96	11
24	AB-24	91	12
25	AB-25	92	10
26	AB-26	94	10
27	AB-27	93	08
28	AB-28	89	15
29	AB-29	89	22
30	AB-30	91	21
31	AB-31	91	20
32	AB-32	91	19
33	AB-33	97	20
34	AB-34	92	19
35	AB-35	91	31
36	AB-36	94	24
37	AB-37	94	15
38	AB-38	91	14
39	AB-39	96	17
40	AB-40	91	21

Table 4.3.4. Grouping pattern of *Alternaria brassicae* isolates on the basis of total number of protein bands and their intensity on SDS-PAGE

Sl. No.	Group	Number of protein bands	Isolate number
1	Group I	up to 10	AB4, AB13, AB22, AB27, AB36
2	Group II	10 - 20	AB1, AB2, AB3, AB6, AB7, AB8, AB10, AB11, AB15, AB16, AB18, AB19, AB21, AB23, AB25, AB26, AB28, AB31, AB33, AB35, AB37, AB38, AB39, AB40
3	Group III	> 20	AB5, AB9, AB12, AB14, AB17, AB20, AB24, AB29, AB30, AB32, AB34

Table 4.3.5 Details of polymorphism obtained from the selected primers used in RAPD analysis of *Alternaria brassicae*

Sl. No.	Primer	Sequence (5' – 3')	Total no of bands	Monomorphic bands	Polymorphic bands	Percentage of polymorphism
1	OPA 3	AGTCAGCCAC	4	0	4	100
2	OPA 4	AATCGGGCTG	8	0	8	100
3	OPA 5	AGGGGTCTTG	4	0	4	100
4	OPA 6	GGTCCCTGAC	9	4	8	88.8
5	OPA 7	GAAACGGGTG	9	2	7	77.7
6	OPA 8	GTGACGTAGG	6	1	5	83.3
7	OPA 9	GGGTAACGCC	6	0	6	100
8	OPA 13	CAGCACCCAC	7	6	6	85.7
9	OPA 14	TCTGTGCTGG	10	0	10	100
10	OPA 15	TTCCGAACCC	9	0	6	66.6
11	OPA 16	AGCCAGCGAA	10	0	10	100
12	OPA 17	GACCGCTTGT	9	1	8	88.8
13	OPA 18	AGGTGACCGT	10	7	7	70
14	OPA 19	CAAACGTCGG	11	0	11	100
15	OPA 20	GTTGCGATCC	11	2	9	81.8
16	OPC 3	GGGGGTCTTT	8	0	8	100
17	OPC 4	CCGCATCTAC	9	0	9	100
18	OPC 5	GATGACCGCC	7	0	7	100
19	OPC 7	GTCCCGACGA	6	1	5	83.3
20	OPC 8	TGGACCGGTG	8	0	8	100
21	OPC 9	CTCACCGTCC	9	1	8	88.8
22	OPC 10	TGTCTGGGTG	9	7	6	66.6
23	OPE 2	GGTGCGGGAA	9	0	9s	100
24	OPE 3	CCAGATGCAC	10	0	10	100
25	OPE 4	GTGACATGCC	8	4	6	75
26	OPE 5	TCAGGGAGGT	10	0	10	100
27	OPE 6	AAGACCCCTC	8	0	8	100
28	OPE 7	AGATGCAGCC	7	0	7	100
29	OPE 8	TCACCACGGT	9	0	9	100
30	OPE 9	CTTCACCCGA	9	0	8	88.8
31	OPE 10	CACCAGGTGA	8	4	6	75
Total No. of bands			257	43	204	80.3

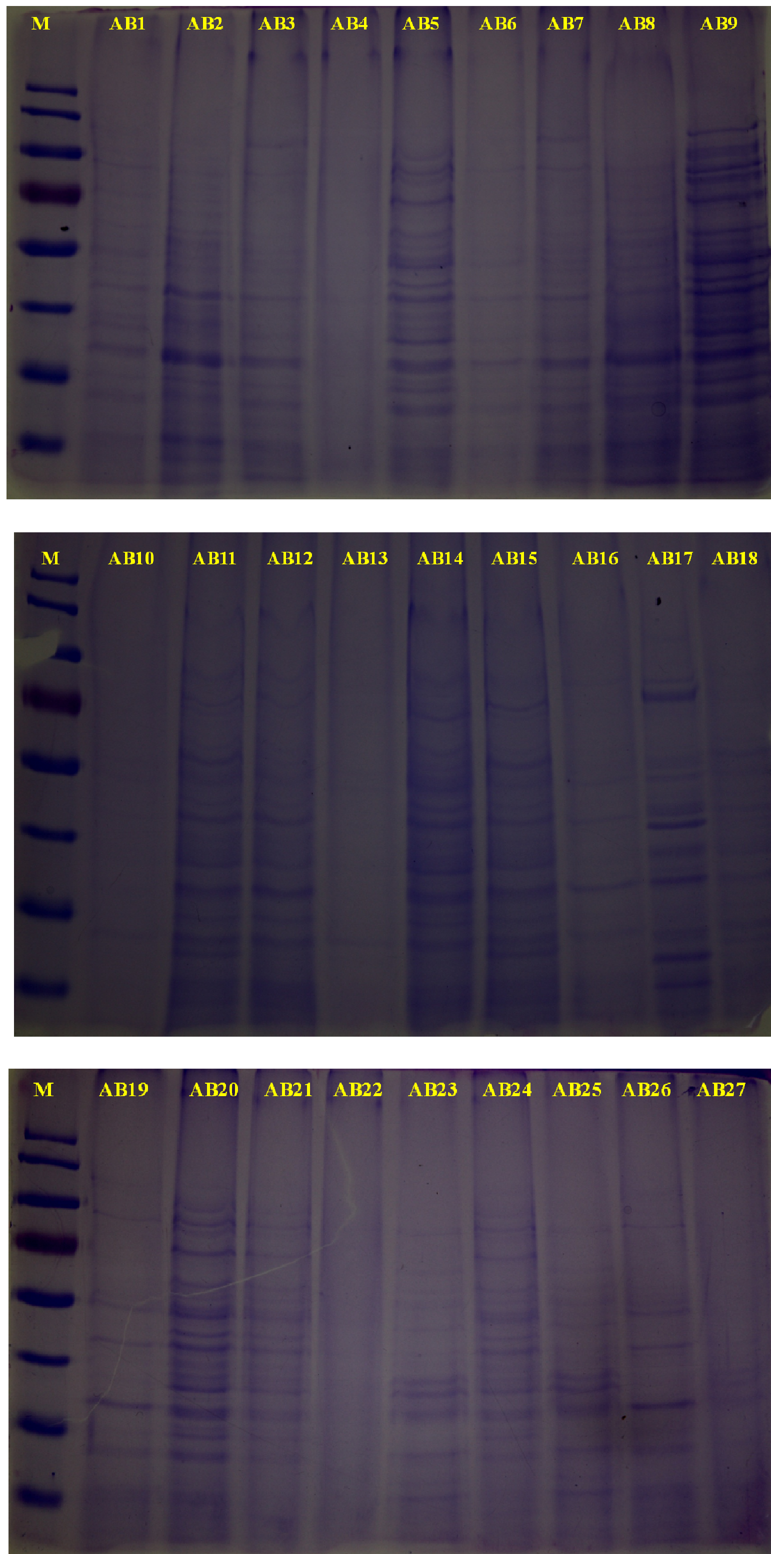
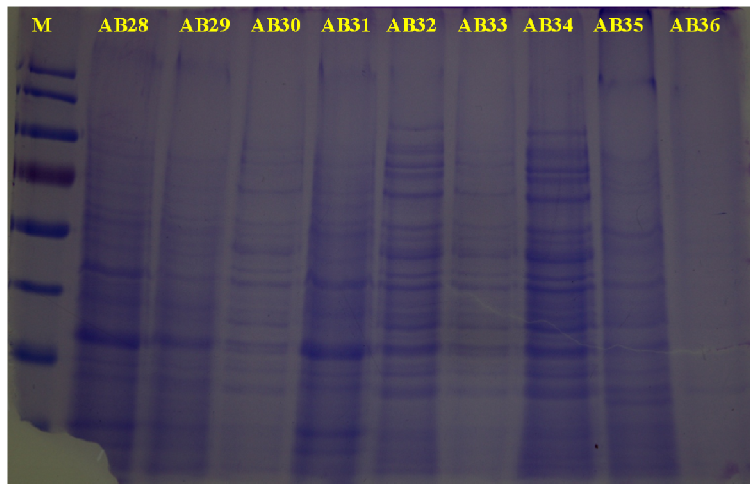


Fig. 4.3.3. Protein profiling of the *Alternaria brassicae* isolates by SDS-PAGE



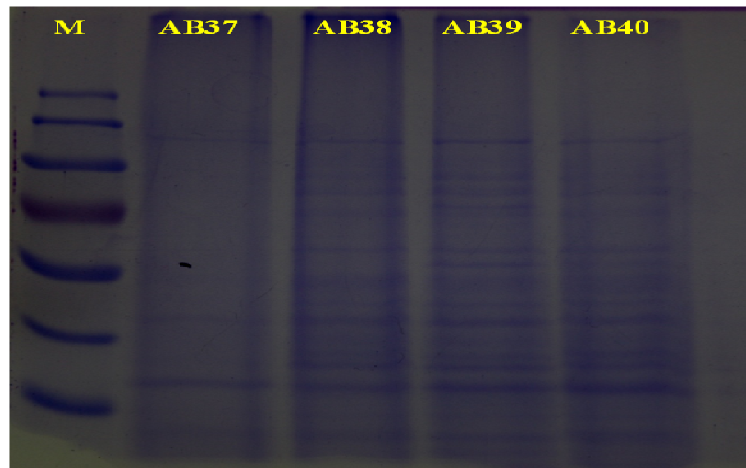
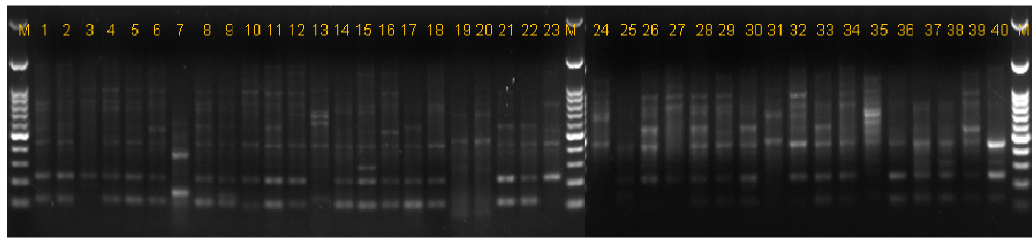
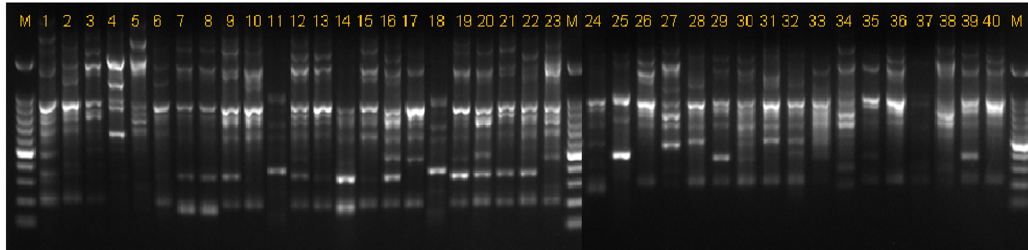


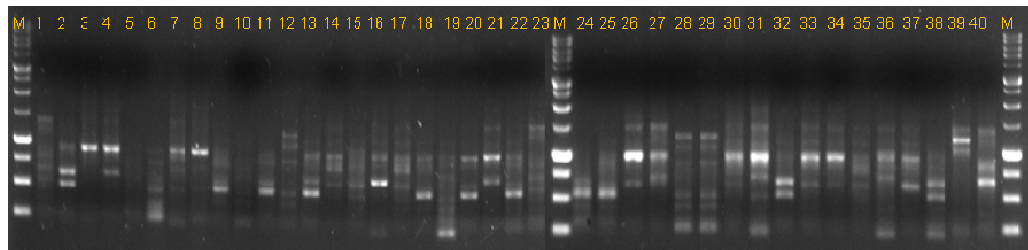
Fig. 4.3.3. Protein profiling of the *Alternaria brassicae* isolates by SDS-PAGE



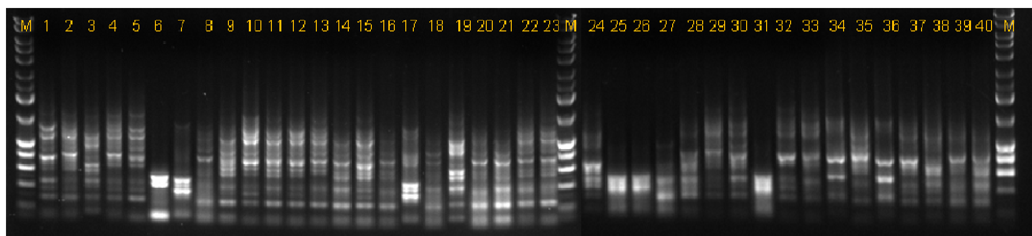
OPE 1



OPE 5



OPC 3



OPA 19

Fig. 4.3.4 Representative gel picture of RAPD profiling of different *Alternaria brassicae* (40) isolates, where M: 100 bp DNA ladder and different numbers denotes different *A. brassicae* isolates.

97 per cent between AB-15 and AB-16 isolates. Majority of the isolates showed more than 80 per cent similarity co-efficient.

The dendrogram constructed through UPGMA analysis clearly demonstrated that the isolates were grouped into six distinct clusters of 0.67 similarity coefficient as shown in (Fig. 4.3.5). First three main clusters had only one isolates each like AB-5, AB-7 and AB-6, the forth cluster contains three isolates namely AB-24, AB-25 and AB-26. The fifth cluster consists of AB-1, AB-2, AB-3, AB-4, AB-27, AB-28, AB-29, AB-30, AB-31, AB-32, AB-33, AB-35, AB-34, AB-36, AB-37, AB-39 and AB-40. The remaining isolates forming sixth cluster.

Analysis of ITS nucleotide sequence data

Nucleotide sequence data for ITS1 and ITS 4 was obtained by using accessions obtained through NCBI. All distance values were calculated using the Kimura 2-parameter distance algorithm (Mega 4.1 software). The nucleotide sequence of ITS1, 5.8S rDNA and ITS2 region of 40 isolates of the pathogen varied from 510-590bp (Fig.4.3.6). It was largest (583) in AB -17 whereas the smallest 513bp in length in AB-15 isolates. The ITS sequence of these isolates showed 56 to 100 per cent similarity with each other (Fig.4.3.7). Phylogram generated by neighbor joining method clustered ten *A. brassicae* isolates into two major clusters. Cluster I comprised three sub cluster AB-38, AB-39, AB-36, AB-35, AB-34, AB-33, AB-32, AB-31, AB-30, AB-29, AB-28, AB-27, AB-26, AB-22, AB-11, AB-9 and AB-25 forming first sub cluster, AB-10, AB-23 and AB-24 forming second sub cluster and isolate AB-17 formed third sub cluster and Cluster II comprised 19 isolates and four sub clusters, AB-16, AB-13 and AB-18 respectively formed first, second and third sub clusters, the remaining isolates AB-12, AB-14, AB-15, AB-1, AB-2, AB-3, AB-4, AB-5, AB-6, AB-7, AB-8, AB-21, AB-40, AB-37, AB-19 and AB- AB-20 formed fourth sub cluster.

Discussion

Isolates of *Alternaria* sp. differ greatly in respect to cultural, morphological and pathogenic behaviour. Several workers (Vishwanath and Kolte, 1997; Singh *et al.,* 2003; and Tetarwal *et al.,* 2008) have reported cultural, morphological and pathogenic variability among isolates of *Alternaria* spp. In the present investigation biochemical and molecular diversity in various isolates of *A. brassicae* infecting crucifers was observed. The results showed that isolates of *A. brassicae* differ in their characters with respect to enzyme production, protein profile and molecular characters.

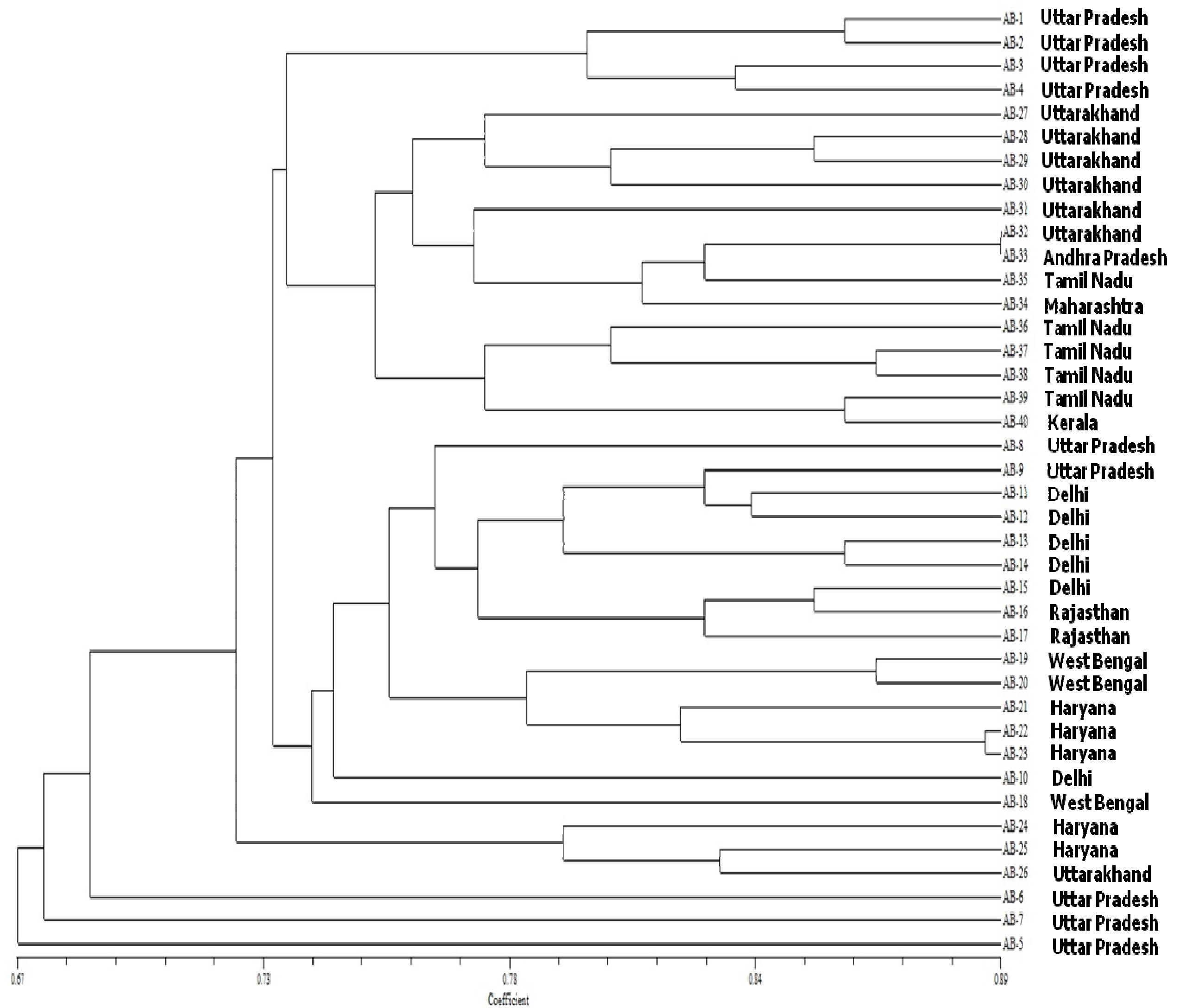


Fig. 4.3.5 Dendrogram derived from RAPD analysis of 20 isolates of *Alternaria brassicae* with 31 primers by UPGMA (Unweighted paired-group method with arithmetic average).

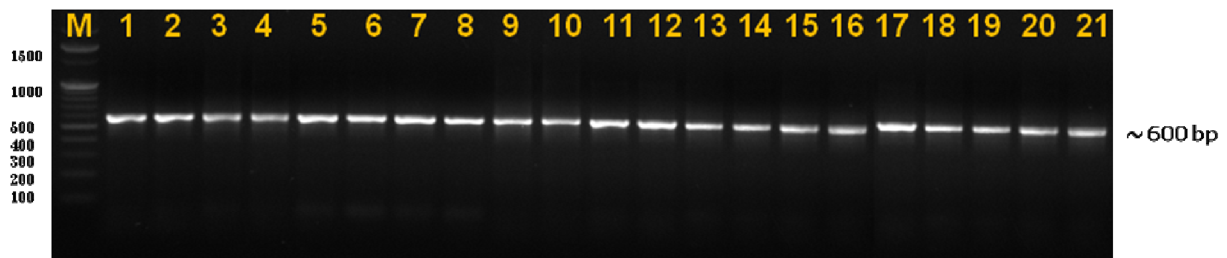


Fig4.3.6. ITS profiles of different isolates of *Alternaria brassicae* where, M: 100bp DNA ladder, Lane 1 - AB-9; Lane 2 - AB-10; Lane 3 - AB-11;Lane 4 - AB-17; Lane 5 - AB-22;Lane 6 - AB-23; Lane 7 - AB-24; Lane 8 - AB-25;Lane 9 - AB-26; Lane 10 - AB-27; Lane 11 - AB-28; Lane 12 - AB-29; Lane 13 - AB-30; Lane 14 - AB-31; Lane 15 - AB-32; Lane 16 - AB-33; Lane 17 - AB-34; Lane 18 - AB-35; Lane 19 - AB-36; Lane 20 - AB-38; Lane 21 - AB-39

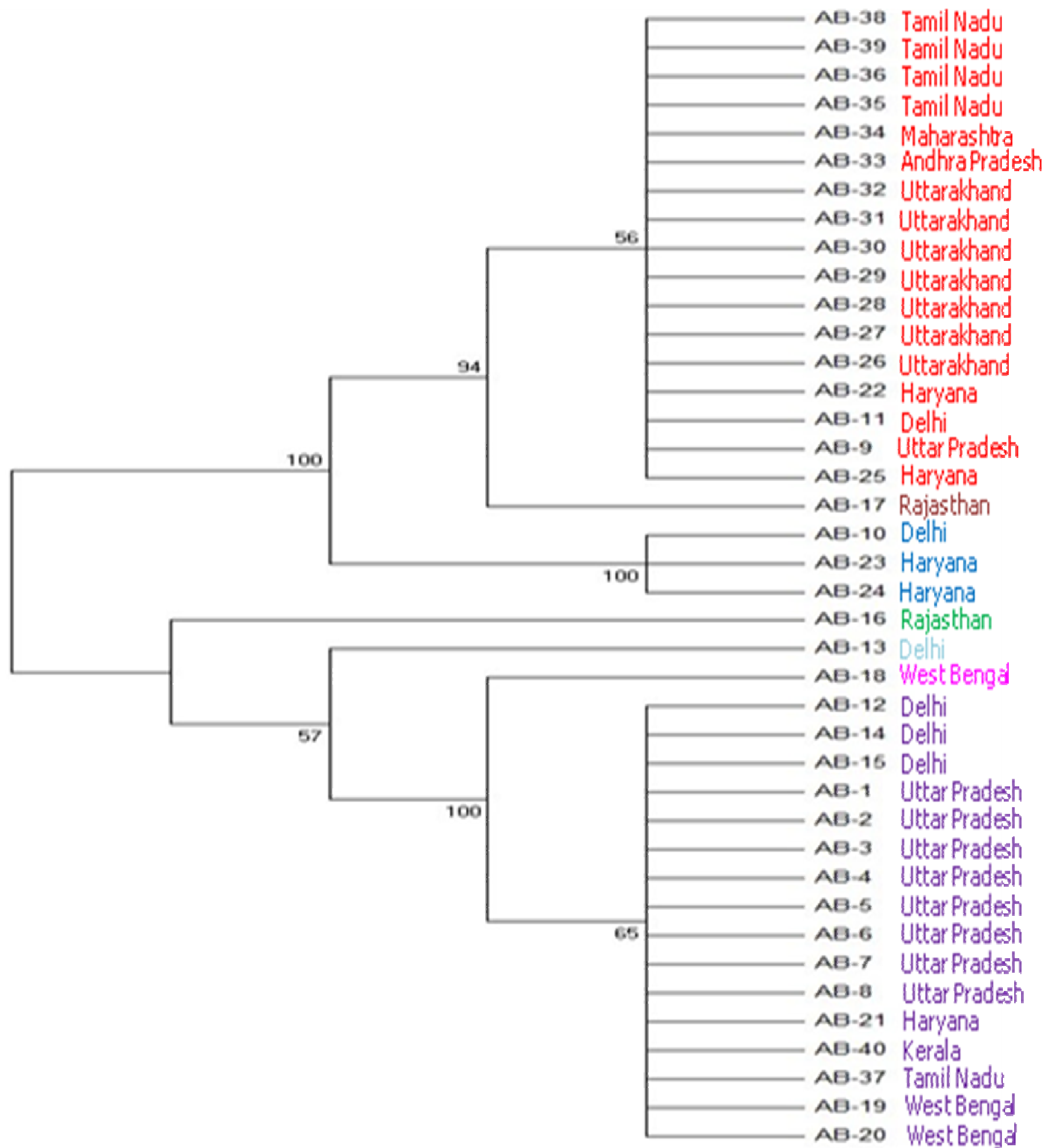


Fig. 4.3.7 Phylogenetic relationship among 40 isolates of *Alternaria brassicae.* Dendrogram was based on ITS1-5.8s-ITS2 region nucleotide sequence aligned using Mega 4x algorithm. Bootstrap –Neighboring Joint (NJ) analysis was done with 100 replicates and values at nodes of branches refer to number of times the branching is supported

Enzyme production potential was varied widely and there were some correlation with disease severity of the particular isolate in mustard, cabbage and cauliflower also. The isolates shown more vigor in cross infectivity normally produced more pectinse than lipase and cellulase. This showed the importance of pectinase over cellulose and lipase. The brassinin had some role in spore germination inhibition. The protein profiling had no correlation with RAPD and ITS markers. Invention of PCR techniques, molecular diagnosis and classification of plant pathogens has greatly influenced the epidemiological and disease management studies. The molecular biology techniques have added to the analysis of variability in those organisms where stable morphological markers were absent.

The genetic diversity at the molecular level of the 40 isolates of *A. brassicae* from diverse locations of India revealed different behaviour while using RAPD (Morris *et al*., 2000; Wang and Zhang, 2003 and Weir *et al*., 1998) and nucleotide sequence of ITS region. The grouping pattern of these isolates could be correlated with the geographical locations from which the *A. brassicae* isolates collected in RAPD marker analysis. Eleven isolates of *A. solani* from the same region of India are genetically distinct (Kumar *et al.,* 2008) in which, the isolates originating from southern part of India comprises of Tamil Nadu and Karnataka were found together in the clustering pattern. However, the isolates from Maharashtra, Uttar Pradesh and Rajasthan were more diverse and formed two or more clusters indicating the genetic diversity in these region. Likewise some isolates from Maharashtra, Uttar Pradesh, Rajasthan were very similar and came in a single cluster. Similarly, in case of *A. brassicicola,* Bock *et al*. (2002) reported that multiple isolates collected from particular locations tends to cluster together, implying the potential for population structure and moderate levels of genetic diversity existing within isolates. The ITS region has been used extensively in fungal taxonomy. It was also reported to show variation between and within the species (Nazar *et al*., 1991). Forty isolates of *A. brassicae* phylogenetic analysis of nucleotide sequence of ITS region showed that there is no correlation between clustering pattern and their geographical origin. Among the two techniques used to identify the polymorphism within *A. brassicae* revealed that RAPD marker gave more polymorphism than ITS region. The possible reason for higher polymorphism in RAPD marker may be attributed to more number of primers used in the study which covers more loci in the genome. Results obtained in this study showed that rapid analysis of variation in *Alternaria brassicae* is possible with the RAPD method. It has also been suggested that RAPD analysis could detect single base

changes, i.e. point mutations or insertion of single base pairs (Williams *et al*., 1990). Peever *et al*. (2000) reported that there was no correlation between RAPD cluster analysis and pathogenic abilities in some of the isolates of *Alternaria* species pathogenic to citrus. Similar results have been reported by Sharma and Tiwari (1998) *A. brassicicola* isolates were genotypically different although they were similar on the basis of morphological and cultural characteristics.

5. GENERAL DISCUSSION

Progression of Alternaria leaf spot was found to be influenced by environmental factors like minimum temperature, maximum temperature, relative humidity etc. prevalent under field conditions. The progress of the disease was found to be highly varied among the crops and cultivars. Gupta *et al.* (2003) tested three varieties of mustard against Alternaria blight and found that significant variation in disease index. Maximum temperature showed positive correlation with Alternaria blight severity and minimum temperature did not showed higher correlation, which is not matching with earlier findings of Awasthi and Kolte (1994). Singh *et al.* (2009) also found the positive correlation between maximum, minimum temperature and disease index. But Meena *et al.* (2011) found that maximum temperature positively influenced Alternaria blight severity and minimum temperature had less influence.

In the present study a relationship between atmosphere temperature and relative humidity and infection and disease progression was studied. In 2010-2011, the influence of maximum temperature in Alternaria blight development was also varied between crop and season. The correlation between the maximum temperature and Alternaria blight was higher in case of 2010-2011. And the influence of minimum temperature was lower and no significant difference was seen between 2010-2011 and 2011-2012. Morning (maximum) RH has less influence on Alternaria blight severity with its correlation coefficient ranges from 0.09 to -0.01 in 2010-2011. But in 2011-2012 it ranges from -0.5 to -0.43. So the influence of morning relative humidity varies with the weather and cultivar used. In both the years, the influence of afternoon relative humidity influences Alternaria blight severity. Its correlation coefficient ranges from -0.38 to -0.44. Singh *et al.* (2009) also recorded negative correlation between disease index and weather factors like relative humidity I and II. In present study mustard shown higher susceptibility and followed by cauliflower and cabbage. This clearly indicates that not only the weather parameter but also physiological and biochemical composition of plant as well as plant age plays a major role in plant disease development (Meena *et al*., 2004).

The Alternaria blight development highly depends on spore germination and incubation period. Nowicki *et al.* (2012) reported that the optimal spore germination temperature ranges from 18-24°C and relative humidity >90 per cent. In the present study also the optimal spore germination temperature for *A. brassicae* was recorded as 20-24°C, although spores could

germinate at a wide range of temperature (10-30°C). This shows that the pathogen has adaptability to wide range of temperature. High air humidity (90-100 % RH) is a critical for the *A. brassicae* infection (Humpherson-Jones and Phelps, 1989). In the present study, *A. brassicae* spores germinated at all levels of relative humidity tested. But higher levels were more favorable. The present study indicated the influence of weather factors on disease development. The correlation coefficient showed that there might be some other factor(s) involved in the disease development, which requires further studies. This information becomes a useful guide for resistence selection of crops and crop cultivars among crucifers and efficient management of pathogen.

Alternaria brassicae isolates differ greatly in respect to cultural, morphological and pathogenic behaviour. Several workers (Goyal *et al*., 2011, Singh *et al.,* 2007) have reported cultural, morphological and pathogenic variability among isolates of *Alternaria* spp. In the present investigation cultural, morphological and pathogenic diversity in various isolates of *A. brassicae* infecting crucifers was observed. The results showed that isolates of *A. brassicae* differ in their colony characters, colony diameter and sporulation. Most of the isolates of *A. brassicae* showed different mycelia pigmentation on PDA, CDA and CLEA. The colour varied from whitish to black. Ramegowda (2008), also noticed different pigmentation on isolates of *Alternartia* spp. Other colony characters such as texture, culture margin and appearance of mycelial growth were highly varied among the isolates. The culture characters also revealed the difference in zonation pattern of the isolates on PDA, CDA and CLEA. This type of variations in zonation may be due to the lack of essential nutrition for growth of *A. brassicae* isolates on PDA and CDA. Environmental causes such as pH, temperature, relative humidity, quantity of medium might have influenced the colour, zonation, margin shape and sporulation pattern of different isolates (Humpherson-Jones and Phelps, 1989).

The spore production of forty isolates on PDA media mentioned above showed variation in sporulation pattern. The isolate AB-18 produced higher (3.90×10^{4} / ml) number of spores than other isolates. Most of the isolates depicted high growth rate and high number of spore production on CLEA followed by PDA and CDA. Umamaheshwari *et al.* (2008), also observed same pattern in *Alternaria alternata* and *A. cumcummerina* on Watermelon Leaf Extract media. Colony diameter, colony colour, texture, spore morphology, sporulation pattern on different medium showed high variation among forty isolates tested. These variations were not according

to the geographical location and plants (Cabbage, cauliflower and mustard). Earlier authors also reported the variability in *Alternaria* spp. associated with different crop plants showing non lineage with samples collected from different geographical regions.

All the isolates were found pathogenic in nature against their respective host. The reaction of different isolates on set of fifteen differential varieties revealed that all the differentials were susceptible to different isolates of *A. brassicae* on differentials each isolate behaved differently. The finding of the present study clearly indicates the existence of pathogenic variability in *A. brassicae.* Awasthi and Kolte (1989) distinguished three isolates A, C and D from Pant Nagar on the basis of pathogenesis on crucifers. Mehta *et al.* (1997) had also indicated that different isolates collected from different agro-climatic zones of India behaved differently on the set of seventeen host differentials. Wide variation in virulence among the races of *A. brassicae* have also been observed by Saharan and Kadian (1983) though studies were based on isolates collected from different species of rapeseed and mustard from same location. In the present investigation the isolates were collected from different agro-climatic regions of India and shown greater differential reactions which strongly suggest existence of variability in *A. brassicae*.

Isolates of *Alternaria* spp. differ greatly in respect to cultural, morphological and pathogenic behaviour. Several workers (Vishwanath and Kolte, 1997; Singh *et al.,* 2003; and Tetarwal *et al.,* 2008) have reported cultural, morphological and pathogenic variability among isolates of *Alternaria* spp. In the present investigation biochemical and molecular diversity in various isolates of *A. brassicae* infecting crucifers was observed. The results showed that isolates of *A. brassicae* differ in their characters with respect to enzyme production, protein profile and molecular characters.

Enzyme production potential varied widely and there were some correlation with disease severity of the particular isolate in mustard, cabbage and cauliflower also. The isolates showed more vigor in cross infectivity normally produced more pectinase than lipase and cellulose. This shows the importance of pectinase over cellulose and lipase. The brassinin had some role spore germination inhibition. The protein profiling had no correlation with RAPD and ITS markers. Invention of PCR techniques, molecular diagnosis and classification of plant pathogens has greatly influenced the epidemiological and disease management studies. The molecular biology

techniques have added to the analysis of variability in those organisms where stable morphological markers were absent.

The genetic diversity at the molecular level of the 40 isolates of *A. brassicae* from diverse locations of India revealed different behaviour while using RAPD (Morris *et al*., 2000; Wang and Zhang, 2003 and Weir *et al*., 1998) and nucleotide sequence of ITS region. The grouping pattern of these isolates could be correlated with the geographical locations from which the *A. brassicae* isolates collected in RAPD marker analysis. Eleven isolates of *A. solani* from the same region of India are genetically distinct (Kumar *et al.,* 2008) in which, the isolates originating from southern part of India comprises of Tamil Nadu and Karnataka were found together in the clustering pattern. However, the isolates from Maharashtra, Uttar Pradesh and Rajasthan were more diverse and formed two or more clusters indicating the genetic diversity in these region. Likewise some isolates from Maharashtra, Uttar Pradesh, and Rajasthan were very similar and came in a single cluster. Similarly, in case of *A. brassicicola,* Bock *et al*. (2002) reported that multiple isolates collected from particular locations tends to cluster together, implying the potential for population structure and moderate levels of genetic diversity existing within isolates. The ITS region has been used extensively in fungal taxonomy. It was also reported to show variation between and within the species (Nazar *et al*., 1991). Forty isolates of *A. brassicae* phylogenetic analysis of nucleotide sequence of ITS region showed that there is no correlation between clustering pattern and their geographical origin. Among the two techniques used to identify the polymorphism within *A. brassicae* revealed that RAPD marker gave more polymorphism than ITS region. The possible reason for higher polymorphism in RAPD marker may be attributed to more number of primers used in the study which covers more loci in the genome. Results obtained in this study showed that rapid analysis of variation in *Alternaria brassicae* is possible with the RAPD method. It has also been suggested that RAPD analysis could detect single base changes, *i.e.* point mutations or insertion of single base pairs (Williams *et al*., 1990). Peever *et al*. (2000) reported that there was no correlation between RAPD cluster analysis and pathogenic abilities in some of the isolates of *Alternaria* species pathogenic to citrus. Similar results have been reported by Sharma and Tiwari (1998) *A. brassicicola* isolates were genotypically different although they were similar on the basis of morphological and cultural characterstics.

7. SUMMARY AND CONCLUSION

Dark leaf spot *(Alternaria brassicae*) is one of the important diseases in crucifers *viz.*oil seed rape, cabbage, cauliflower and mustard causing serious yield and quality loss in production due to the seed borne nature of the pathogen which have been reported in many countries throughout the world including India. This species have the ability to survive in seeds for several months at different temperatures and relative humidity. Till date no resistance was found against the disease. For understanding of epidemiology, host–pathogen co-evolution, and resistance management, knowledge of plant pathogen variation is required. Severity of Alternaria blight on brassicas differs among seasons and regions as also between individual crops within a region. This may be due to existence of variability among isolates of Alternaria species. In the present study, we have studied the relationship between atmosphere temperature and relative humidity and infection and disease progression by *A.brassicae* on different crucifers. Maximum and minimum temperature showed wide variation on disease appearance while morning pH had no significance variation. Among the crucifer crops used, mustard shown higher susceptibility and followed by cauliflower and cabbage. Along with physiological parameters the Alternaria blight development highly depends on spore germination and incubation period. In the present study the optimal spore germination temperature for *A. brassicae* was recorded as 20-24°C, although spores could germinate at a wide range of temperature (10-30°C). This showed that the pathogen has adaptability to wide range of temperature. *A. brassicae* spores were found to be germinated at all levels of relative humidity tested. But higher levels were more favorable. The study indicated the influence of weather factors on disease development.

Cultural, morphological and pathogenic diversity in various isolates of *A. brassicae* infecting crucifers was also observed. The results showed that isolates of *A. brassicae* differ in their colony characters, colony diameter and sporulation. Most of the isolates of *A. brassicae* showed different mycelia pigmentation on PDA, CDA and CLEA. The colour varied from whitish to black. Most of the isolates depicted high growth rate and high number of spore production on CLEA followed by PDA and CDA. Colony diameter, colony colour, texture, spore morphology, sporulation pattern on different medium showed high variation among forty isolates of *A.brassicae* tested. These variations were not according to the geographical location and

plants (Cabbage, cauliflower and mustard). The pathogenic reaction of different isolates on set of fifteen differential varieties revealed the susceptibility of all the differentials with different behavior of each isolate clearly indicating the existence of pathogenic variability in *A. brassicaze* collected from different agro-climatic regions of India.

Biochemical characterization of the forty isolates of *A. brassicae* showed difference with respect to enzyme production, protein profiling pattern. Enzyme production potential was varied widely and there were some correlation with disease severity of the particular isolate in mustard, cabbage and cauliflower. The isolates shown more vigor in cross infectivity normally produced more pectinase than lipase and cellulase. The brassinin assay showed inhibition of spore germination. Pattern was also varied with rest to each isolates of *A.brassicae*.

Invention of PCR techniques, molecular diagnosis and classification of plant pathogens has greatly influenced the epidemiological and disease management studies. The molecular biology techniques have added to the analysis of variability in those organisms where stable morphological markers were absent.

Forty isolates of *A. brassicae* phylogenetic analysis of nucleotide sequence of ITS region showed that there is no correlation between clustering pattern and their geographical origin. Among the two techniques used to identify the polymorphism within *A. brassicae* revealed that RAPD marker gave more polymorphism than ITS region. The possible reason for higher polymorphism in RAPD marker may be attributed to more number of primers used in the study which covers more loci in the genome. Results obtained in this study showed that rapid analysis of variation in *Alternaria brassicae* is possible with the RAPD method. The protein profiling had no correlation with RAPD and ITS markers.

This present study indicated presence of intra species variation with respect to cultural, morphological, pathogenic nature. Biochemical and molecular characterization confirmed the variability within the isolates from different geographic origin within India.

7. ABSTRACT

Title of the thesis: Epidemiology and diversity of *Alternaria brassicae* (Berk.) Sacc the causal agent of black leaf spot in crucifers.

Black leaf spot caused by *Alternaria brassicae* (Berk.) Sacc. is an important disease of crucifers. The present study was undertaken to evaluate the epidemiology and diversity of *Alternaria brassicae* in different varities of cabbage, cauliflower and mustard. Disease progression studies under field conditions revealed that there was periodical increase in lesion number and Per cent Disease Index (PDI). PDI progression was higher in mustard varieties followed by cauliflower and cabbage varieties. The speed of progression of disease among the crop cultivars was calculated by using Area Under Disease Progress Curve (AUDPC) and apparent rate of infection (*r*-value). Highest AUDPC value was recorded on Pusa Bold variety of mustard and the lowest was recorded on CJ-182 variety of cabbage. This may enable to select the slow disease progressing varieties for the management of the disease. Relative humidity was found negatively correlated with the development of disease. On the contrary, maximum and minimum temperature was positively correlated with disease development. A total of forty isolates were collected from different parts of the country and its cultural, morphological and pathogenic variability were studied. All the isolates showed high level of variability *in vitro* in respect to mycelia growth, growth pattern and sporulation. Higher growth rate and high number of spore production were recorded on Cauliflower Leaf Extract Agar (CLEA) followed by Potato Dextrose Agar (PDA) and Czepak Dox Agar (CDA). Substantial variation was found in spore morphology in respect to conidial length, width and number of septa. Detached leaf technique was carried out on fifteen varieties and it revealed that all these isolates behaved differently on differentials. The Per Cent Disease Index (PDI) varied from 0-100 per cent. The studies, therefore, indicated the existence of variability among isolates of *Alternaria brassicae*. High level of variability was observed in respect to enzyme production and most of the isolates produced more cellulase than lipase and pectinase. The pectinase production also has been correlated with disease development. Brassinin hydrolase has played an important role in anti-fungal activity which inhibits the spore germination, growth and development. Protein profiling studies revealed that the isolates were grouped into three distinct groups. Among the two techniques used to identify the polymorphism at molecular level, Randomly Amplified Polymorphic DNA (RAPD) marker given more polymorphism than the Internal Transcribed Spacer (ITS) region.

क्रूसीफर्स में कृष्णपर्ण धब्बा उत्पन्न करने वाले *आल्टरनेरिया ब्रैसिकी* (बर्क) सैक का हेतु विज्ञान एवं विविधता

सार

आल्टरनेरिया बैसिकी (बर्क) सैक द्वारा उत्पन्न कृष्णपर्ण धब्बा, क्रूसीफेरी कुल के पौधों का एक महत्वपूर्ण रोग है।*आल्टरनेरिया बैसिकी* के हेतु विज्ञान एवं विविधता का पत्तागोभी, फूलगोभी एवं सरसों की विभिन्न किस्मों में मूल्याँकनार्थ प्रस्तुत अध्ययन किया गया। प्रक्षेत्र परिस्थितियों के अन्तर्गत रोग के प्रगति संबंधी अध्ययनों ने दर्शायाकि क्षत–संख्या एवं प्रतिशत रोग घातांक (पी डी आई) में एक आवर्ती बढ़ोतरी थी। सरसों की किस्मों में पी डी आई श्रेढ़ी अधिक थी, तत्पश्चात फूलगोभी एवं पन्तागोभी की किस्मों का स्थान रहा। रोग प्रगतिवक्र के अर्न्तगत क्षेत्र (ए यू डीपी सी) एवं संक्रमण की आभासी दर (आर–नाम) का उपयोग कर फसल की कृषि जीव जातियों के मध्य रोगकारक प्रगति–दर की गणना की गई। सरसों की पूसा बोल्ड किस्म में उच्चतम ए यू डी पी सी मान रेकार्ड किए गए तथा पत्तागोभी की सी जे–182 किस्म में न्यूनतम मान रेकार्ड किए गए। रोग के प्रबंधनार्थ मंद रोग प्रगति वाली किस्मों के वरण में यह अध्ययन सहायक होगा। आपेक्षिक आर्द्रता का रोग के विकास के साथ ऋणात्मक सहसंबंध पाया गया। इसके विपरीत, अधिकतम एवं न्यूनतम तापमान, रोग के विकास के साथ धनात्मक रूप से सहसंबंधित पाए गए। देश के विभिन्न भागों से कुल चाली सविलग एकत्र किए गए और *आ॰ बैसिकी* की संवर्धन संबंधी, आकारिकीय एवं रोग जनकता संबंधी परिवर्तनशीलता का अध्ययन किया गया। कवक जाल वृद्धि–ढ़ग एवं स्पोर निर्माण के संदर्भ में, सजीव कोशिकाओं के बाहर प्रयोगशाला में सभी विलगों ने उच्च–स्तरीय परिवर्तनशीलता दर्शायी। फूलगोभी पत्ती एक्सट्रैक्ट अगार (सी एल ई ए) संवर्धन माध्यम पर अधिक वृद्धि दर एवं बीजाणुओं का अधिक संख्या में बनना रेकार्ड किया गया, तत्पश्चात पोटेटो डेक्सट्रौज़ अगार (पी डी ए) एवं जैपेक डॉक्स अगार (सी डी ए) का स्थान रहा। कोनीडिया की लम्बाई, चौड़ाई एवं सेप्टा की संख्या के संदर्भ में स्पोर–आकारिकी में सुस्पष्ट भिन्नता पायी गई। पंदह्र किस्मों पर टूटी पत्ती तकनीक का प्रयोग किया गया। जिसने दर्शाया कि सभी विलगों का परिवर्तितों पर भिन्न–भिन्न व्यवहार होता है। प्रति शत रोग घातांक (पी डी आई) 0–100 प्रति शत की सीमा में था। इस प्रकार से, ये अध्ययन, *आल्टरनेरिया ब्रौसिकी* के विलगों के मध्य विद्यमान परिवर्तन शीलता को दर्शाते हैं। एंजायम–उत्पादन के संदर्भ में उच्चस्तरीय परिवर्तन शीलता देखी गई तथा सभी विलगों लाइपेज़ एवं पेक्टिनेज़ की तुलना में अधिक सेल्यूलोज़ का उत्पादन किया । पेक्टिनेज़ उत्पादन का रोग विकास के साथ सहसंबध भी देखा गया। कवक विरोधी सक्रियता में ब्रैसीनिन हायड्रोलेज़ ने एक महत्वपूर्ण भूमिका निभाई जो स्पोर–अंकुरण, वृद्धि एवं विकास का संदमन करता है। प्रोटीन–प्रोफायलिगं अध्ययनों ने दर्शाया कि इन विलगों को तीन भिन्न–भिन्न समूहों में रखा गया। आण्विक स्तर पर बहुरूपता की पहचान हेतु उपयोग की गयी दो तकनीकों में से यादृच्छिक रूप से प्रवर्धित बहुरूपी डी एन ए (आर ए पी डी) चिह्न के इंटरनल ट्रॅांसक्राइब्ड स्पेसर (आई टी एस) रीजन की तुलना में अधिक बहुरूपता प्रदान की।

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APPENDIX

Potato Dextrose Agar (PDA) medium

Peeled potato: 200g

Dextrose : 20g

Agar- agar : 20g

Distilled water: 1000ml

Carrot Potato Agar (CPA) medium

Peeled potato: 100g

Carrot : 100g

Dextrose : 20g

Agar- agar : 20g

Distilled water: 1000ml

Cauliflower Leaf Extract Agar (OLEA) medium

Cauliflower leaf : 200g

Dextrose : 20g

Agar- agar : 20g

Distilled water: 1000ml

Nutrient Agar (NA) medium:

Beef Extract: 3.0g

Peptone : 5.0g

Agar-Agar : 20g

Distilled Water: 1000ml

Minimal synthetic media

$MgSO_4 . 7H_2O$: 0.2g

K_2HPO_4 : 0.9g

KCl : 0.2g

NH_4NO_3 : 1.0g

$FeSO_4.7H_2O$: 0.002g

$MnSO_4$: 0.002g

$ZnSO_4$: 0.002g

Carbon source (Cellulose for cellulase, pectin for pectin and olive oil for lipase)

Agar : 20g

Distilled Water: 1000ml

DNA Extraction buffer

1M Tris. HCl, pH 8.0

1.5M NaCl

0.01 M EDTA

2per cent CTAB

Bradford Reagent - Bradford reagent can be made by dissolving 100 mg Coomassie Blue G-250 in 50 ml 95 per cent ethanol, adding 100 ml 85 per cent (w/v) phosphoric acid to this solution and diluting the mixture to 1 liter with water.

Table. Preparation of test samples for the Bradford protein assay.

Test Sample	Sample volume (µl)	Water (µl)	Bradford reagent, µl
Blank	0	200	800
BSA Standard (10 µg/ml)	20	180	800
BSA Standard (20 µg/ml)	40	160	800
BSA Standard (30 µg/ml)	60	140	800
BSA Standard (40 µg/ml)	80	120	800
BSA Standard (50 µg/ml)	100	100	800
Protein Sample	100	100	800

DNA Extraction Protocol:

1. Weigh of 1-1.5 g of mycelium
2. Mycelia were grinded into a fine powder using liquid nitrogen and a pre chilled pestle and mortar
3. Fine powder transferred to a 30 ml centrifuge tube and 4 ml of pre warmed extraction buffer added and gently mixed by inverting
4. Tubes were heated in 65°C for 1 hr by inverting every 10 min
5. Tubes were taken out from water bath and after cooling, equal volume of phenol: Chloroform: Isoamyl Alcohol (25:24:1) was added and mixed slowly
6. Centrifuge was done @ 13000 rpm for 20 min at room temperature
7. Supernatant was transfer to a new tube and equal volume of Chloroform: Isoamyl Alcohol (24:1) was added, gently mixed again centrifuged at same conditions
8. Supernatants was taken out and 0.6 volume of chilled isopropanol was added to precipitate DNA and kept overnight at -20°C
9. DNA was transferred to eppendorf tube and centrifuge @ 10000 rpm for 10 min at 4°C
10. Supernatant discarded and pellet washed by 500 micro litre 70 per cent ethanol 2-3 times
11. Pellet was dried in room temperature and dissolved in 100-200 micro litre TE buffer. DNA was quantified by using 0.7per cent Agarose gel electrophoresis

Gel extraction using QIA quick gel extraction kit:

1. The DNA fragment, excised from the agarose gel with a clean, sharp scalpel and the size of the gel slice was minimized by removing extra Agarose.
2. The gel slice was weighing in a colourless tube and 3 volume of buffer QG was added to one volume of gel (*i.e.* 100 mg 100µl)
3. To completely dissolve the gel slice, it was incubated at 50°C for 10 min (to help dissolve gel, every 2-3 min during the incubation, mixed by vortexing the tube).
4. After the gel slice was dissolve completely, the colour of the mixture checked, it was yellow (similar to buffer QG without dissolved agarose)
5. One gel volume of chilled isopropanol was added to the sample and mixed
6. QLA quick spin column placed in a provided 2 ml collection tube

7. To bind DNA the sample applied to the QLA quick column and centrifuged for 1 min. (Maximun 800µl sample was added and for sample of more the 800µl, simply loaded and spined again)
8. Discarded flow through and QLA quick column back in the same collection tube
9. Again 0.5 ml of buffer QG was added to QLA quick column and centrifuged for 1 min
10. To wash, 0.75 ml of buffer PE was added to QLA quick column and centrifuged for 1 min
11. Discarded flow through and centrifuged the QLA quick column for on additional 1 min at 13000 rpm
12. QLA quick column placed into a clean 1.5 ml micro centrifuge tube
13. To elute DNA, 30-50µl of buffer GB (10mM Tris- HCl, pH-8.0) was added to the center of the QLA quick membrane and centrifuged the column for 1 min.
14. To analyse purified DNA, 1 volume of loading Dye was added to 5 volume of purified DNA and loaded on 0.8 per cent agarose gel.

Ligation Protocol:

The PCR amplified DNA fragments were ligated pGEM-T Easy vector.

The ligation mixture was as follows:

2x rapid ligase buffer	5µl
pGEM-T Easy vector	1µl
DNA ligase (2U/µl)	1µl
PCR amplified DNA	3µl
Total	10µl

The ligation mixture was incubated at 4° C for 16-18 h. The ligation was taken for transformation

Preparation of competent cells:

The competent cells were prepared by calcium chloride method as described by Mendel and Higa (1970).

- 50ml Broth (LB) was inoculated with overnight grown culture of DH 5a stain of *Escherichia coli* (Stratagene) and incubated at 37°C for 1.5 h with constant shaking at 200 rpm in a shaker incubator till the bacterial growth as measured by optical density reached 0.3 OD at 600nm
- The culture was then aseptically transferred to 30 ml sterile screw capped tubes and kept on ice for 20min
- The cells were centrifuged at 5000 rpm for 10 min at 4°C
- The cells were resuspended gently in 5ml ice cold 0.1M $MgCl_2$ solution and centrifuged at 5000 rpm for 10 min at 4°C
- The last step was repeated once again
- The pellet was resuspended in 10ml cold 0.1M $CaCl_2$ solution and kept on ice for 1 hr
- The cells were recovered by centrifuging at 5000 rpm for 10 min at 4°C and the pellet were resuspended in 1 ml of chilled 0.1 M $CaCl_2$ and used for transformation after keeping on ice for 1hr

Transformation of competent cells:

- 200µl competent cells were added to 10µl of the ligation mixture in a sterile microfuge tube. Gently mixed and kept on ice for 1 hr
- The bacterial cell-DNA mixture was given a heat shocked at 42°C for 2 min. 800µl of LB medium was added and transformants were allowed to grow at 37°C for 1 hr in shaker incubator at 200 rpm
- 200ul of cell suspension were aseptically plated either such for after concentration of Luria Agar (LA) plated containing ampicillin, X-gal and IPTG (100µl of 1 percent ampicillin, 200µl of 2 per cent X-gal and 20ul of 0.1 IPTG for 100ml LA). (The plat were incubated overnight at 37°C)

Selection of transformation:

The transforms were selected on the basis of blue/white colony colour. The white colonies were selected and subsequently plated on IXA (IPTG-X-gal-Ampicillin) plates. This plate having individual transformants in grid served as master plate.

Rapid screening for the recombinant clones having inserted:

White recombination bacterial colonies may be formed either due to insertion of DNA fragment or due to even a small deletion in the lacZ operon of vector itself. To know which with colonies have the insert a rapid screening method (Sambrook *et al.*, 1989) was used which was as follows:

- A small amount of overnight grown bacterial colonies were picked from master grid individually with the help of sterile tooth pick and mixed with 50µl of 10m MEDTA (pH 8.0) in sterile microfuge tubes. One or two blue colonies of PGEM-T Easy transformed bacterial cells were also taken for control
- 50µl of fresh lysis solution (2N NaoH, 0.5 per cent SDS, 20 per cent sucrose) was added in each tube and vortexing was done for 30 second which caused rapid disruption of bacterial cell wall
- The mixture was incubated at 70°C for 5 min and was cooled down to room temperature
- 1.5µl of 3M KCL and 0.5µl of 0.4per cent of bromophenolblue were added to each tube and vortexing was done for 30 seconds
- The mixture was then incubated for 5 min on ice and centrifugation was done at 1000 rpm for 30 min for 4° C in table top centrifuge
- Bacterial cell debris was removed and 30µl of supernatant from each tube along with the control were electrophoresed in 0.7 per cent agarose gel
- The lanes which show higher plasmid band than the control was considered as recombinant plasmid and the respective colonies were taken for isolatoion of recombinant plasmid DNA

Isolation of Recombinant Plasmid DNA by Miniprep Method:

Isolation of recombinant plasmid DNA was done by modified alkaline lysis method (Brinboim and Doly, 1979)

- Selected white colonies which were presumed to contain DNA fragment insert in rapid screening were individually inoculated 2ml of LB medium containing ampicillin (50µg/ml) in sterile capped culture tubes
- Tubes were then incubated overnight at 37°C at 200 rpm in a shaker incubator
- The overnight grown bacterial cells were then transferred to 1.5ml sterile eppendorf tubes and cells were harvested by centrifuging in a table top centrifuge for 1 min care was taken to remove the medium adhering to the cell pellet

- The pellet was resuspended in 100µl of solution and mixed vigorously by vortexing
- Then 200µl of freshly prepared lysis solution II was added and mixed gently
- 150µl of ice cold solution III was then added and mixed gently with lysed cell suspension and the mixture was kept on ice for 15min
- The chromosomal DNA and the bacterial cell debris were removed by centrifugation at 15000 rpm for 15 min at 4°C in a table top centrifuge
- The supernatant was taken and equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) was added. It was vortexed well, centrifuged in a table top centrifuge for 15 min at room temperature
- A clear phase was transferred to a fresh eppendorf tube
- The DNA in aqueous phase was precipitated by adding 0.8 volume of isopropanol, and kept on ice for 10min
- The mixture was centrifuged at 15000 rpm for 20 min at 4° C
- To the pellet 200µl of 70 per cent ethanol was added the tube was rotated well so that pellet from the wall gets suspended in 70 per cent alcohol. DNA was then pelletized by centrifuging at 15000 rpm for 5 min
- The pellet was finally suspended in 30µl of sterile double distilled water

Restriction analysis:

Selected recombinant plasmid was subjected to digestion with restriction enzymes and incubated at 37°C for 2 hr. *EcoR*I restriction was done at know the size of the insert.

The reaction conditions are as follows:

Plasmid DNA (500-1000ng)	3.0µl
10x reaction buffer	2.0 µl
Restriction enzyme (5U/ µl)	1 µl
Sterile double distilled water	13.0 µl
Total	20 µl

After restriction, the DNA samples were electrophoresed in 1per cent agarose gel. Fragment Sizes were assessed in comparison with 1kb DNA marker.