Variability in *Alternaria mali* (Roberts) and Management of Alternaria Leaf Blotch of Apple through Systemic Acquired Resistance Activators

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Dedicated
TO THE CAUSE OF PLANT PATHOLOGY

Sher-e-Kashmir
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Certificate – I

This is to certify that the thesis entitled **"Variability in *Alternaria mali* (Roberts) and Management of Alternaria Leaf Blotch of Apple through Systemic Acquired Resistance Activators"** submitted in partial fulfillment of the requirements for the award of the degree of **Doctor of Philosophy in Agriculture (Plant Pathology),** to the Faculty of Postgraduate Studies, Sher-e-Kashmir University of Agricultural Sciences and Technology of Kashmir, is a record of bonafide research work carried out by **Tariq Ahmad Sofi (Regd. No. 2006-154-D)** under my supervision and guidance. No part of the thesis has been submitted for any other degree or diploma.

It is further certified that information received during the course of investigation has duly been acknowledged.

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We, the members of the Advisory committee of **Tariq Ahmad Sofi (Regd. No. 2006-154-D)**, a candidate for the degree of **Doctor of Philosophy in Agriculture (Plant Pathology)**, have gone through the manuscript of the thesis entitled, **"Variability in *Alternaria mali* (Roberts) and Management of Alternaria Leaf Blotch of Apple through Systemic Acquired Resistance Activators"** and recommend that it may be submitted by the student in partial fulfillment of the requirements for award of the degree.

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ABSTRACT

Seven districts of the valley were surveyed to record the status of Alternaria leaf blotch disease of apple. The disease was prevalent in all the districts surveyed with an overall disease incidence and intensity of 42.11 and 21.68 per cent, respectively. Highest disease incidence and intensity was recorded in district Pulwama and least in district Shopian. Pathogenic behaviour of twenty one isolates of *Alternaria mali*, isolated from apple leaves of cv. Red Delicious, was established following Koch's postulates. Morpho-cultural and molecular studies were made to ascertain the prevalence of variability among the isolates. Colonies varied in their cultural behaviour ranging from velvetty to cottony, mostly appressed, with regular to irregular margins. Colour of the colonies ranged between light to dark olivacious. Growth rate of isolates was between 5.86 to 8.21 mm with

fast growth in Am-13 and least in Am-5. Isolates impregnated media with colour ranging between grey to brown. Morphological studies of isolates revealed variations in their colour, size, shape and septations of hyphae, conidiophore and conidia. Significant variations were observed in conidiophore and conidial septations. Average conidial size varied from 21.36-31.74 µm x 8.34-14.48 µm. Isolates when tested for their virulence revealed variation in incubation period and number and size of the lesions produced. Isolate Am-1 was categorized as most virulent. The dendrogram analysis based on cultural, morphological and pathogenic studies revealed high diversity within *Alternaria mali* population. At 67 per cent similarity matrix all the isolates formed 2 clusters with 12 isolates in cluster I and 9 in cluster II. However dendrogram on molecular (RAPD) basis revealed 5 clusters at 68 per cent Dice similarity coefficient. There was no congruence between the RAPD pattern and cultural, morphological and pathogenic characters. Isolates that were identical for one spectrum were often dissimilar for other spectrum. Commercially important apple cultivars were screened for resistance against *Alternaria mali* and disease resistance of variable magnitude was observed in available germplasm. White Dotted Red was highly resistant and American Apirouge resistant in reaction. Red Delicious and Red Gold were highly susceptible. All the Systemic Acquired Resistance (SAR) chemicals tested against the disease significantly lowered the disease as compared to check. β-aminobutyric acid (BABA) at a concentration of 2000 ppm was most effective with least disease intensity before and after pathogenic inoculation. The other SAR chemicals in decreasing order of their efficacy were tri-potassium phosphate (K_3PO_4), benzothiadiazole S-methyl ester (BTH), 2,6-dichloroisonicotinic acid (INA), calcium hydroxide ($Ca(OH)_2$), di-potassium ortho-phosphate (K_2HPO_4) and calcium carbonate ($CaCO_3$). Penconazole used as standard check proved superior to all the SAR activators, except BABA.

Key words: Apple, *Alternaria mali*, Alternaria leaf blotch, Variability, Varietal screening, SAR Activators

Signature of Student

Dated: 20-06-2011

Signature of Major Advisor

Dated: 20-06-2011

Acknowledgement

In the name of "ALLAH" Most gracious, Most merciful

Research is an evolving concept. Any endeavor, in this regard is challenging as well as exhilarating. It implies the testing of our nerves. It brings to light our patience, vigour and dedication. Every result arrived at is a modest beginning for a higher goal and no work can be termed as a one-man show. It needs the close cooperation and guidance of experts in the field to achieve something worthwhile and substantial.

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INTRODUCTION

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INTRODUCTION

The apple (*Malus domestica* Borkh.) is the most ubiquitous of temperate fruits cultivated in Europe and Asia from antiquity. It was known to the Greeks and Romans and mentioned by Theophrastus in the 3rd century B.C. Since then the apple has been distributed into almost all parts of the world. It is a premier table fruit, rich in carbohydrates, proteins, minerals like calcium, phosphorus, iron, potassium, thiamine and vitamin $B_{6.}$

Leading apple producing countries in the world are China, USA, Poland, Iran and Turkey with present world production close to 60 million tons (FAO, 2009). The apple ranks fourth in fruit crop after citrus, grapes and banana worldwide. India ranks 7th in the apple with an annual production of 19,85,000 MT (FAO, 2009). The most widely grown cultivars are 'Golden Delicious', 'Delicious' and its red sports. In India apple was introduced by British in Kullu Valley of Himachal Pradesh (H.P) back in 1865, and is predominantly grown in Himalayan states like Jammu and Kashmir (J&K), H.P. and Uttaranchal which account for about 90% of the total production of country. The productivity has touched 12 m tons per

hectare which however is low as compared to some other developed countries (Anonymous, 2002).

Apple being major fruit crop of J & K has attained the status of an industry. It is grown on an area of 1,20,541 hectares in the state with an annual production of 13,15,197 metric tonnes that constitutes about 94 and 86 per cent of the fresh and total fruit production of the State respectively (Anonymous, 2009).

Like other horticultural crops apple is attacked by several pathogens which impair the quality and quantity of the fruit. However, huge losses of the crop are incurred mostly by fungal diseases. The major fungal diseases include scab, Alternaria leaf blotch, powdery mildew, collar rot, root rot, sooty blotch and fly speck etc. Amongst these, Alternaria leaf blotch caused by *Alternaria mali* is prevalent in all the apple growing areas of world and is one of the economically important apple diseases. *Alternaria mali* was first described in 1924 in the United States by J. W. Roberts and has become a problem in the southeastern United States. The disease assumed an alarming threat to the crop because of premature defoliation in North Carolina and has potential of becoming important throughout the apple producing regions wherever susceptible cultivars strains of Delicious are grown (Filajdic and Sutton, 1991). An outbreak of the disease was recorded

in Japan as early as in 1956 and its occurrence corresponded to increased cultivation of highly susceptible cultivar ‘Starking Delicious’, a red-sport of Delicious. Alternaria blotch has already attained the status of economically important disease in Japan and other Asian countries (Sawamura, 1990). In India, Alternaria leaf blotch of apple was first reported by Gupta and Agarwala (1968) from H.P. In J&K, the occurrence of disease (*A. mali*) was reported by Shahzad *et al*. (2002). In the state the disease previously considered to be of minor significance has now attained the status of major disease (Anonymous, 2000) and is prevalent in almost all apple growing orchards of Kashmir valley with a potential threat to existing apple plantation.

Alternaria leaf blotch fungus chiefly affects the foliage and manifests as greyish-brown lesions and in severe case lead to premature defoliation. Initial symptoms of the disease appear on the leaves in late spring to early summer as small circular, light brown to brown or blackish spots which gradually enlarge in diameter. Periodic change in lesions lead to characteristic silvery grey lesions impregnated with blackish tiny mass of spore. Secondary expansions of the spots lead to coalescing of lesions, resulting in the formation of large irregular necrotic patches. Leaves with infected petioles turn yellow and defoliate prematurely.

One of the significant aspects of the biology of an organism is the morphological and physiological characters of an individual within a species which are not fixed. This holds true with fungi also, although is not frequent in asexually produced individuals of the progeny. Variability studies are important to document the changes occurring in the population and individuals with variability in morphological and physiological traits indicate existence of different pathotypes. The variability is a well known phenomenon in genus *Alternaria* and such variability may be seen in spore shape and size, growth and sporulation, pathogenicity, etc. Diversity appears even in single spore isolates. Filajdic and Sutton (1992) reported variability in virulence among isolates of *Alternaria mali.* Shahzad (2003) while recording the disease status reported Alternaria leaf blotch of apple prevalent in all the districts of Kashmir with varied degree of incidence and intensity and even recorded the variation in susceptibility among different apple cultivars. This variation may be attributed to many factors including variability in pathogen. Understanding pathogen population structure and mechanisms by which variation arises within a population is of paramount importance for devising a successful disease management programme. Thus requires continuous monitoring of development of the pathogen variability more so for the breeding programmes aimed at developing resistant genotypes to the given set of pathogenic races (Pastor *et al*., 1998;

Sartorato, 2002). Variation in pathogen populations can generally be detected with methods like morphological, cultural, pathogenic and molecular specificity. DNA markers have become a powerful tool to study taxonomy and molecular genetics of a variety of organisms. The random amplified polymorphic DNA (RAPD) allows quick assessment of genetic variability, and has been used to study inter and intraspecific variability among isolates of several fungal species.

Cultivation of resistant varieties is most favoured approach to reduce the cost of cultivation, risk of development of resistance in pathogen, risk to human health and environmental pollution, therefore cultivation of apple varieties resistant to Alternaria blotch is the most effective and favourable method of controlling the disease. As such development of disease-resistant cultivars has a high priority in most apple breeding programmes throughout the world (Kellerhals and Furrer, 1994; Lespinasse *et al*. 2000). There is tremendous pressure on apple industries in most developed countries to produce high quality fruit with minimum use of agricultural chemicals. However, development of resistant varieties requires the collection of information on resistance and/or susceptibility level of cultivated cultivars. Such information is also key to development of strategy for integrated control of the disease.

Among the various disease management strategies available, chemical application still dominates our approach involving frequent fungitoxicant interventions throughout the world (Katan *et al*., 1983; Koller *et al*., 2004). Kashmir valley with temperate environmental conditions is a favourable place for the development of fungal diseases and to combat these diseases a need-based spray schedule comprising 6-8 sprays of fungitoxicants is in vogue. However, the development of fungicide resistance in pathogenic populations is one of the most serious constraints because substantial changes in the populations of several major plant pathogens in their sensitivity to fungicides have been observed, leading frequently to significant crop damage and forcing either discontinuation or modification in the use of important chemicals. The frequent and successive use of fungitoxicants, particularly systemic, is likely to develop resistance among the pathogen and as such threaten the success of spray programme (Lelancette *et al*., 1987; Koller *et al*., 2004) due to the occurrence of resistance in the pathogen (Koller and Wilcox, 1999). Some isolates of *Alternaria mali* have developed tolerance to fungicides like iprodione, mancozeb and captan (Lee and Kim, 1986; Osanai *et al*., 1987; Asari and Takahashi, 1988). To counter the problem, eco-friendly approaches to the disease management are the need of hour. Use of systemic acquired resistance (SAR) activators is reported to be environmentally benign

compared to current pesticides (Vallad and Goodman, 2004). Unlike traditional pesticides, SAR activators do not exhibit any direct antimicrobial activity, and they provide a way to control disease without asserting direct selective pressure on pathogen population. SAR is based on multiple natural defense mechanisms, and this makes it less likely that a pathogen can readily develop resistance to this control measure. Systemic acquired resistance is broad spectrum plant defense response that can be induced biologically by challenging a plant with a weaker strain of a specific pathogen or exposing a plant to natural and/or synthetic chemical compounds. In SAR number of defenses like, oxidative burst, defensive molecules (peroxidase, PR-proteins), lignifications, cell wall cross linking etc. are activated locally and are also induced systemically against further infection by the pathogen.

Since the crop and disease are of paramount importance to the state (J&K) and no studies on pathogen variability and development of management strategy through eco-friendly approaches (like SAR activators) have been conducted in the State, therefore the present study was conducted with the following objectives:

- To asses disease status in Kashmir valley
- To ascertain prevalence of variability in *A. mali* in Kashmir valley

- To screen commercially important apple cultivars against the most virulent isolate of the pathogen
- To evaluate the efficacy of Systemic Acquired Resistance (SAR) activators for management of the Alternaria leaf blotch disease

CHAPTER - 2

REVIEW OF LITERATURE

CHAPTER – 2

REVIEW OF LITERATURE

In this chapter, attempt has been made to review the research conducted in India and abroad on the aspects pertaining to the problem under investigation. For the sake of convenience, the text of the literature has been reviewed under the following heads:

2.1 Status of the disease

Alternaria blotch caused by *Alternaria mali* Roberts, is one of the most important diseases of apple worldwide especially in Japan, South Korea, China, Zimbabwe, Yugoslavia and other Asian countries (Jones and Aldwinkle, 1990) and reportedly has great potential of becoming an alarming disease in other apple producing regions including United States (Filajdic and Sutton, 1992; Bulajic *et al*., 1996). The disease has recently been reported from Turkey (Ozgonen and Karaca, 2006). *A. mali* was first identified in United States in 1924 (Roberts, 1924). The disease received attention after its outbreak in Japan in 1956 (Sawamura, 1972). Hoshino and Sawamura (1970) and Sawamura (1972) proposed the name Alternaria blotch to the disease caused by a pathogenic strain of *A. mali*.

In India, the disease was first reported from Himachal Pradesh in 1968

(Gupta and Agarwala, 1968), whereas the occurrence of leaf blight disease on apple by *A. tenuissima* dates back as early as 1987 (Puttoo, 1987). Shahzad *et al*. (2002) reported the occurrence of Alternaria leaf blotch caused by *A. mali* in apple in Jammu and Kashmir.

Yoon *et al*. (1989a) reported outbreak of leaf blotch of apple initiating in late May in Daeugu area of Korea, with rapid increase in disease intensity in late July to reach the highest levels in late August. They further reported yearly fluctuations in disease intensity however, with no change in order of disease in the cultivars. Filajdic and Sutton, (1991) reported 0 to 95 per cent disease incidence in 60 apple orchards of Western North Carolina (USA) during 1989 and 1990, with 23.33 to 30.00 per cent orchards having at least 10 per cent disease incidence. They observed the strains of Delicious (Classic Red, Red Delicious, Silver Spur, Oregon II and Red Chief) to be more susceptible to disease. Bulajic *et al*. (1996) recorded 50 per cent defoliation in "Red Delicious" apple cultivar due to *A. mali* in some of the orchards of Yugoslavia. In Kashmir average disease incidence and intensity of 41.32 and 20.47 per cent has been recorded with highest disease incidence and intensity of 43.50 and 22.57 respectively, observed in district Anantnag (Shahzad, 2003).

2.2 Pathogenicity

Various methods have been employed by many workers to establish the pathogenicity of the fungus on apple. Tweedy and Powell (1962) sprayed 240 fruits and 31 leaves of six succulent Jonathan apple shoots with a spore suspension of *A. mali* and observed the lesions development after 5 to 10 days on 20 leaves of four shoots and 217 fruits. Injuring detached leaves with a bundle of needles soaked in a spore suspension of *A. mali* was the most effective method of inoculating adult apple trees, and seedlings with 4-7 leaves were best inoculated by spraying with the spore suspension (Saito *et al*., 1975). Ozgonen and Karaca (2006) also proved the pathogenicity of *A. mali* by inoculating wounded detached, apple leaves with a conidial suspension (10^6 conidia / ml). Johnson *et al*. (2000) however tested different isolates of *A. alternata* including apple pathotype for pathogenicity using a leaf necrosis bioassay.

2.3 Variability

Two types of *A. mali* with distinct morphology have been reported by Roberts (1924) with type 'A' having sparse aerial mycelium with abundant olivacious conidial production in a dark carpet-like mycelial mass on the surface of the medium and type 'B' having abundant gray aerial mycelium with sparse conidial production. Further, variation was also

observed in conidial measurement with average size of type 'A' conidium being 26 × 9 µm and that of type 'B' conidium being 49 × 9 µm. Identification of some *Alternaria* species especially those which produce host-specific toxins is extremely difficult due to their high degree of variability which extends even to non-pathogenic isolates (Johnson *et al*., 2000). Perusal of literature reveals variable colony characteristics of *A. mali* cultured on similar media. For instance, an *A. mali* isolate from Yugoslavia produced dark mycelial mat on PDA (Bulajic *et al.*, 1996), whereas *A. mali* isolated from Kashmir produced initially velvetty white and later turning dark olive gray and finally black colonies on PDA (Shahzad *et al*., 2002). However, Ozgonen and Karaca (2006) reported dark olive circular velvety colonies of *A. mali* on PDA. Filajdic and Sutton (1991) reported that nine isolates of *A. mali* in Western North Carolina (USA) significantly varied in terms of conidial length. Eight isolates of *A. mali* from Western North Carolina (USA) were reported to be pathogenic on Delicious seedlings of apple with varied virulence (Filajdic and Sutton, 1992). Previous research has provided evidence for the existence of variability among the *Alternaria* isolates. Preliminary reports on variability in *Alternaria* species were made from Holland (Van Schreven, 1953) and UK (Mridha, 1983). According to Mridha (1983), thirteen isolates of *A. brassicae* tested on selected cultivars of winter oilseed rape differed in their virulence. Diversity in morpho-

cultural and physiological characteristics among different isolates of *Alternaria* spp. infecting potato, tomato, Bt-cotton and sesame have been reported by several workers (Kaul and Sexena, 1988; Perez and Mortinez, 1996; Naik *et al.,* 2006; Ramegowda and Naik, 2008).

Isolates of *A. brassicae* showed distinct variability in their growth and colony characters, size and shape of conidia, conidiophore, sporulation and formation of chlamydospores (Patni *et al*., 2005). Michereff *et al*. (2003) reported a high variability among 38 isolates of *A. brassicicola* associated with crucifers in Pernambuco, Brazil. Thrall *et al*. (2005) reported substantial variation in growth rates of *A. brassicicola* isolates, as well as in pathogen populations and noticed strong differences in the average size of lesions formed by individual pathogen isolates. Similarly, Kolte *et al*. (1989, 1991) and Awasthi and Kolte (1989) also reported variability in *A. brassicae*. Koul and Saksena (1989) found that the spore dimensions were extremely variable and not only exhibited variability between the cultures of the different strains but also between the cultures of the same strain. Kumar *et al*. (2003) reported variability among isolates of *Alternaria brassicae* in terms of conidial length, breadth and number of septation. The average conidial length varied from 118.62 to 194.52 µm and breadth from 14 to 23 µm. However, horizontal and vertical septation

varied from 3 to 12 and 0 to 6 respectively. Mehta *et al*. (2003) reported variability in isolates of *Alternaria brassicae* regarding conidial length, breadth and septation. Further, the incubation period on detached leaves varied from 3-13 days.

The races have been defined on the basis of their cultural characteristics, dimensions of spores and virulence (Bonde, 1929; Neergaard, 1945). Even pathogenic differences were found among isolates originating from different germ tube tips from the same conidium (Stall, 1958). Rotem (1966) found great variation in cultural characteristics (colour, growth and sporulation) among various isolates of *A. solani*, thus giving rise to the claims of the existence of races. He reported a wide variability in spore dimensions of forty two isolates of *A. solani*.

Bond (1929) demonstrated large variation in *Alternaria* isolates from potato tubers, with respect to colony morphology, sporulation capacity, and growth rate on artificial media as well as in pathogenicity. Henning and Alexander (1959) obtained preliminary evidence for physiological differences in *Alternaria solani* isolates. Kaul and Saxena (1988) reported four cultural groups of *Alternaria solani* which differed from each other in cultural and physiological characters. Similarly Kumar *et al*. (2008) reported significant variation in cultural characters, pigmentation and

growth rate of *A. solani*, four isolates were having circular margin with smooth surfaced colony and six isolates were with irregular margin. The pigmentation varied from yellow, brown, black, brownish to greenish black on potato dextrose agar medium. Varma *et al*. (2006) found variability in conidial morphology (size of conidia and number of septa) among isolates of *Alternaria solani*, the causal agent of early blight of tomato. The average size of conidia varied from 150-224.9 µ x 12.4-17.2 µ. The number of horizontal (4-14), vertical (0-3) and beak (0-8) septa also varied among the isolates. Babu *et al*. (2000) recorded variability among *Alternaria solani* isolates with regard to virulence, radial growth etc.

Tetarwal *et al*. (2008) observed cultural, morphological and pathogenic variability in different isolates of *A. alternata* infecting senna plants. Slavov *et al*. (2004) reported diversity in colour of colonies in a single spore isolates of *Alternaria alternata* tobacco pathotype.

Out of 80 isolates of *Alternaria burnsii*, Pipliya and Jadeja (2008) found 32 isolates dark brown to black, 19 isolates typical black, 20 isolates olive green and 9 isolates with dirty white colonies.

Kumar (2004) recorded considerable variability in pathogenicity among the isolates of *Alternaria triticina*. He found significant difference in size of conidia, colour and growth pattern of the isolates. Das and Narain

(1990) found variability in virulence among the isolates of *Alternaria triticina* without any morphological differences between them. Kumar and Rao (1980) found difference in pathogenicity among the isolates of *Alternaria triticina* and *Alternaria tenuis* and proposed that it may be the result of differences in metabolism and biochemical properties.

Ziman *et al*. (1998) observed colour variation of different isolates of *Sclerotinia sclerotiorum* which varied from white to brown. Price and Colhoun (1975), Basha and Chatterjee (2007) and Ghosolia and Shivpuri (2007) also observed variation in isolates of *S. sclerotiorum* to cause infection in various hosts.

Cultural variation has also been observed in other fungal pathogens viz., *Macrophomina phaseolina* (Gaur *et al*., 2004) and in *Phomopsis vexans* (Suman and Sugha, 2004). Sharma *et al*. (2004) reported marked variation in cultural characters of *Macrophomina phaseolina* isolated from pearl millet, sesame, horsegram and mothbean crops. Even various isolates of *Macrophomina phaseolina* obtained from different tissues of the same host species vary in their cultural, morphological and pathological characteristics (Kulkarni and Patil, 1966; Khare *et al*., 1973; Byadgi and Hedge, 1988). Shekhar *et al*. (2006) found seven isolates of *Macrophomina phaseolina* varied in their cultural characteristics and pathogenic behaviour.

On the basis of colony colour, they divided the isolates into four groups i.e. grayish white, blackish gray, dark black in centre with cremish periphery and cottony white colour. They observed Hyderabad isolate to be most virulent while Coimbatore isolate was least virulent. Kumar and Sugha (2004) found that 37 isolates of *Phomopsis vexans* vary in their colony colour, type, and time taken for formation of pycnidia, sporulation density and ability to cause pre- and post-emergence seedlings damping-off, although morphological and cultural variations in isolates of *Phomopsis vexans* have long been recognized (Panwar and Chand, 1968; Munatanola *et al*., 1985; Islam and Pan, 1990).

Lal and Kandhari (2009) studied variability in 25 isolates of *Rhizoctonia solani* causing sheath blight of rice and found variation in colony colour, colony diameter and hyphal width, while on the basis of growth pattern they categorized the isolates into three groups and found 8 isolates with abundant growth, 4 isolates moderate and 13 isolates with slight growth. Sunder *et al*. (2003) reported that colony colour of *Rhizoctonia solani* ranged from brown, light brown, dark brown and yellowish brown.

Lima *et al.* (1998) tested three isolates of *Lasiodiplodia theobromae* and found variation in lesion development on susceptible cultivar.

Similarly, Jadeja (1991) also reported that the four isolates of *L. theobromae* were infective but differed in virulence. Bhatt and Jadeja (2008) found that 23 isolates of *L. theobromae* varied in the colony characters viz, colony colour, type, growth habit and pigmentation as well in the ability to incite fruit rot in mango. They found 19 isolates with dark brown colour and 4 with typical black colonies.

Cardoso *et al*. (2007) reported variability among *Penicillium* isolates with regard to colony morphology and conidia coloration.

Fifteen isolates of *Neovossia indica* significantly differed in morphological traits like size, shape and colour of mycelia and sporidia (Selvamani and Singh, 2009).

2.4 Molecular variability

The random amplified polymorphic DNA (RAPD) analysis is extremely powerful method that allows quick assessment of genetic variability in various taxa, and has been used to study inter- and intra-specific variability among isolates of several fungal species (Tigano-Milani *et al*., 1995; Gherbawy, 1999, 2001; Inglis *et al*., 2001; Gherbawy and Abdelzaher, 2002). It provides comprehensive information regarding the genetic variability among the pathogen populations as it is based on the entire genome of an organism (Achenback *et al*., 1997). RAPD markers

have been used to study intra- and inter-specific variations in genus *Alternaria* (Cooke *et al*., 1998; Weir *et al*., 1998; Roberts *et al*., 2000).

Genetic variability in *A. solani* isolated from different parts of plants and different locations has been studied using RAPD (Weir *et al*., 1998; Morris *et al*., 2000; Wang and Zhang 2003). Peever *et al*. (2002) found no correlation between RAPD clustering for the tested isolates and pathogenicity. Further, their cluster analysis of RAPD revealed significant differentiation between USA, Colombia, Turkey, South Africa, Israel and Australian isolates of *A. alternata*. Tigano *et al*. (2003) observed genetic variability among the isolates collected from the same geographical region and similarity between the isolates from the different geographical regions. Gherbawy (2005) concluded that strains of a single species may significantly vary in their RAPD patterns and no correlation exists between RAPD groups and pathogenicity against tomato. Varma *et al*. (2006) found high genetic variability among the isolates of *A. solani* by RAPD profiles but no evidence for geographical clustering of isolates with high level of genetic similarity was reported suggesting that isolates were widely spread across India. Based on preliminary screening of 86 random primers Kumar *et al*. (2008) selected four random primers to study genetic diversity within 11 isolates of *A. solani,* because of their reproducible results of

polymorphism. They found no effect of location of isolation; rather two isolates from two different provinces were closer to each other. Further, cluster analysis resulted in two main clusters at 30 percent similarity coefficient. However, the groupings based on RAPD data could not be correlated to the ones based on morphology and pathogenicity.

Adachi *et al*. (1993) demonstrated considerable variability within ribosomal RNA genes of just one pathotype of *A. alternata* from pear orchards in Japan and classified 271 isolates of *A. alternata* into eight different types. Inconsistency in the level of variability within this species was confirmed by Kusaba and Tsuge (1994). High levels of genetic variability have been found in apparently asexual populations of *F. oxysporum* (Gordon and Marlyn, 1997). Weir *et al*. (1998) and Morris *et al*. (2000) reported high level of genetic diversity among the isolates of tomato black mould pathogen (*A. solani*) by RAPD analysis. Morris *et al*. (2000) reported high level of variation among isolates collected from a single species by RAPD analysis. Tigano *et al*. (2003) reported RAPD analysis as an efficient method for detection of genetic variability in *A. cassiae* and *A. alternata* isolates occurring in *Senna obtusifolia* (sicklepod), and also for distinguishing *Alternaria* species. Gherbawy (2005) used four different RAPD primers to study genetic diversity of nine *Alternaria* species

represented by 27 isolates and reported genetic similarity between isolates of *A. alternata* species-group ranging from 14.8 to 100 per cent, whereas it ranged between 14.81 to 96.30 per cent among other species evaluated in the study. He found *A. alternata* from beans clustered together however, the level of genetic diversity fluctuated between 56 and 96.55 per cent. Further, RAPDs were found useful for fungal diagnostic purposes as they produce usually distinct fingerprints for each isolate and for each species. Shah *et al.* (2010) found isolates of *Botryodiplodia theobromae* quite variable at the genetic level by RAPD analysis.

RAPD analysis by Roberts *et al*. (2000) revealed that the isolates of *A. alternata* and *A. tenuissima* separate into different groups during cluster analysis, whereas they did not separate in the study by Pryor and Michailides (2002). Based on 18s RNA sequencing data, *A. alternata*, *A. brassicae* and *A. raphani* formed a strong clade of very closely related sister taxa (Jasalavich *et al*., 1995). Cooke *et al*. (1998) studied 13 phytopathogenic *Alternaria* species by using RAPD patterns, each species producing a distinct pattern of DNA fragments, including a high degree of similarity among isolates of the *Alternata* species-group, but he reported that *A. raphani* did not group with *A. brassicae* in his phylogenetic tree. RAPD results of Gherbawy (2005) showed that *A. brassicae*, *A. dauci*, *A.*

raphani and *A. solani* clustered together in one clade, though previous phylogenetic analysis using sequence data (Pryor and Gilbertson, 2000; Chou and Wu, 2002; Pryor and Michailides, 2002) did not relate *A. raphani* to *A. dauci*.

2.5 Screening of cultivars

Field evaluation of plant material is helpful in identifying the sources of resistance however it is a cumbersome process and subject to uncontrollable environmental conditions (Locke 1948; Foolad *et al*. 2000; Pandey *et al.* 2003). Glasshouse evaluation of seedlings using spray inoculation of a conidial suspension is widely used to screen plant materials (Barksdale, 1969). However, disadvantage of spray inoculation method is that the inoculum may not be uniformly distributed on the leaves. Furthermore, the method is not sensitive enough to discriminate moderately resistant from susceptible plants (Gardner, 1990). An alternative method to obtain more precise and reliable disease data is detached leaf technique (Locke, 1948) in which individual droplets of fungal inoculum suspension are inoculated on leaflets. Detached leaflets are first inoculated with mycelial suspension of *Alternaria solani* in a laboratory assay and disease reaction evaluated using a graded series of lesion diagrams with known diameters (Locke, 1948; 1949). Henning and Alexander (1959) used

'droplet method' to determine the existence of *A. solani* races by inoculating leaflets still attached to plants. Nash and Gardner (1988) applied point inoculation method on a whole plant assay and measured early blight lesion diameter.

On the basis of lesion development on immature fruits, Tweedy and Powell (1962) rated Jonathan and Golden Delicious apples as highly susceptible cultivars to *A. mali* as the lesions appeared after 5 days of inoculation and enlarged up to 9 mm diameter after 30 days. The cultivar Grimes Golden was rated moderately susceptible as symptoms appeared 11 days after inoculation and lesions attained a maximum of 2 mm diameter after 30 days, whereas cultivars Stayman Winesap, Rome Beauty and Winesap were completely resistant. Filajdic and Sutton (1992) evaluated Red Delicious and other few lines against *A. mali* under greenhouse conditions and reported variation in the degrees of susceptibility. Shahzad (2003) evaluated 22 apple cultivars against *A. mali* under natural epiphytotic and found all the cultivars susceptible to the disease with significant variations in disease intensities and accordingly rated White Dotted Red as tolerant, Red Delicious and Early Victoria as highly susceptible. Out of the ten cultivars of apple seedlings screened for susceptibility to *A. mali* in Himachal Pradesh 'Vance Delicious' was highly

susceptible (> 60% disease severity), followed by Oregon Spur and Top Red, while Red Fuji was the least susceptible with < 4% disease severity (Khosla and Sharma, 2005). Shin *et al*. (1986) screened 60 apple cultivars on the basis of natural infection and inoculation against *A. mali* and rated Gala, Honey Gold, Magnolia Gold, Mollies Delicious as highly resistant. Yingzi *et al*. (2000) reported Starking Crimson, Hokuto, Gala and Fuji as most susceptible to Alternaria blotch.

2.6 Systemic acquired resistance (SAR) activators

The role of some external morphological features such as leaf hair density, number of trichomes, number and size of stomata and of some pre-existing and induced chemicals in apple leaves in determining the resistance or susceptibility against Alternaria blotch have been reported by many workers. It is well established that the treatment of plants with various agents (virulent or avirulent pathogen, non-pathogens, cell wall fragments, plant extracts, synthetic chemicals, etc.) can lead to the induction of resistance to subsequent pathogen attack, both locally and systemically. Over the last 30 years a number of compounds (SAR activators) have been shown to be instrumental in enhancing disease resistance or at least decreasing the disease symptoms in plants. SAR activators stimulate plant genes to activate its own defense system to reduce disease infection and

thus increase crop yield (Bishnoi and Payyavula, 2003).

SAR was first described by Ross (1961) using mild strain of tobacco mosaic virus (TMV) to induce systemic resistance of tobacco not only to TMV, but also to other viruses. SAR has since been described in many plant-pathogen interactions of economic importance including tobacco, cucurbits, potato, soybean, tomato, millet, alfalfa, rice, wheat, barley (Kuc, 1982; Kessmann *et al*., 1994) and cacao (Okey and Sreenivasan, 1996) as well as in Arabidopsis (Uknes *et al*., 1992). A number of signaling molecules, including salicylic acid (SA), ethylene, and jasmonic acid (JA), have been shown to amplify and regulate defense responses in plants during initial activation events, such as oxidative bursts or the expression of PR genes (Schenk *et al*., 2003; Lee and Hwang, 2005). SAR developed locally or systemically in response to pathogenic infection or treatment with certain chemicals is effective against a wide range of pathogens and is mediated by a salicylic acid (SA) dependent process (Walters *et al*., 2005).

Various plant activators have been discovered that act at various points in their defense activating networks and mimic all or parts of the biological activation of resistance (Rad *et al.,* 2005). Greenhouse and field experiments have paved the way to the present comprehension of induced resistance as a tool in plant protection (Schönbeck *et al*., 1993; Kessman *et*

al., 1994; Schneider *et al*., 1996; Van Loon *et al*., 1998; Benhamou and Picard, 1999; Tally *et al*., 1999; Cohen, 2001; Kuc, 2001; Bokshi *et al*., 2003; Gozzo, 2003; Soylu *et al*., 2003). Chemically-induced SAR have been found effective against various pathogens such as, *Peronospora tabacina*, *Cercospora nicotianae*, *Phytophthora parasitica* var. *nicotianae*, *Pseudomonas syringae* pv. *tabaci* and TMV (Lyon *et al*.,1995; Strobel and Kuc, 1995; Schneider *et al*., 1996; Kuc, 2001). Plant activators have been successfully used in fields for the control of downy mildew of leafy vegetables, rice blast, tomato spotted wilt virus, various diseases of tobacco and different bacterial diseases (Louws *et al*., 2001; Jones, 2001). Percival *et al*. (2009) assessed the efficacy of three commercially available systemic acquired resistance (SAR) products, Messenger (a.i. Harpin protein), Phoenix (a.i. Potassium phosphite) and Rigel (a.i. Salicylic acid derivative) applied at four different growth stages of tree development (bud break, green cluster, 90% petal fall, early fruitlet) against the foliar pathogens *Venturia inaequalis* and *Venturia pirina* and suggested application of at least three sprays during bud break to early fruitlet formation with the SAR agent which may provide a useful addition to existing methods of apple and pear scab management under field conditions.

In apple, phosphites were reported active against *Phytophthora*

cactorum (Guest *et al.,* 1995), *Dematophora necatrix* and *Venturia inaequalis* (Heaton and Dullahide, 1990). Yoon *et al*. (1989) reported that calcium content of apple leaves showing resistance to *A. mali* was much higher than the leaves susceptible to the disease. Further, foliar spray of calcium compounds (CaO, $Ca(OH)_2$ and $CaCO_3$) inhibited the infection by *Alternaria alternata* f.sp. *mali* in apple. Dibasic and tribasic phosphate salts reportedly induced systemic protection against anthracnose in cucumber caused by *Colletotrichum lagenarium* (Gottstein and Kuc, 1989) and later work demonstrated that broad spectrum disease control was achieved in cucumber using phosphates (Mucharromah and Kuc, 1991). The spray of K_2HPO_4 to rice reduced neck blast caused by the fungus *Pyricularia oryzae* between 29 and 42 per cent, with 12 to 32 per cent increase in grain yield (Mandahar *et al*., 1998).

The non-protein amino acid β-aminobutyric acid (BABA) has been shown to induce broad spectrum resistance in wide range of crops (Jakab *et al*., 2001). In field trials, BABA reduced downy mildew of grapevine (*Plasmopara viticola*) by 57 per cent on cv. Chardonny and by 98 per cent on cv. Cabernet Sauvignon (Reuveni *et al*., 2001). In two-leaf sunflower 50 and 0 pustules per leaf developed in water and BABA treated plants, respectively (Amzalek and Cohen, 2007). Reuveni *et al*. (2003) reported

that DL-β-aminobutyric acids (BABA) and potassium phosphite have capability to control moldy-core in apple fruits under laboratory and field conditions. They observed 40-58 and 100 per cent inhibition in the wounded fruit decay caused by *A. alternata* by 50 and 500 µg/ml of potassium phosphite, respectively. BABA (500 µg/ml) inhibited decay formation by 82 to 90 per cent. Moreover, potassium phosphite and BABA inhibited decay formation even when applied 6 to 48 h post inoculation. Post infection activity of BABA has also been reported in grape against *Plasmopara viticola* (Cohen *et al.,* 1999), in tobacco against *Peronospora tabacina* (Cohen, 1994), in tomato against *Phytophthora infestans* (Cohen, 1994a) and other pathosystems (Cohen, 2002).

MacLennan *et al*. (1963) evaluated various butyric acid derivatives against apple scab and found 2-aminobutyric acid active against the disease. Functional analogs of salicylic acid (SA), such as 2, 6-dichloroisonicotinic acid (INA) and benzothiadiazole S-methyl ester (BTH) have been developed which activate the resistance mechanisms downstream of SA (Kessmann *et al*., 1994; Friedrich *et al*., 1996). ASM (Acibenzolar-S-methyl) has proven effective for the control of several diseases caused by virulent pathogens in the number of plants, including bacterial spot in pepper and tomato (Louws *et al.,* 2001; Romero *et al.,* 2001). Brock *et al*.

(1994) applied 2,6-dichloroisonicotinic acid to the cotyledons of cotton and found next emerging leaves less susceptible to infection by *Alternaria macrospora* than those in control plants. Further, the treatment applied to cotyledons had no visible effect on the development of the plants and had no direct effect on the fungus *in vitro*, suggesting that resistance was induced systemically in cotton plants. Benzo-(1,2,3)-thiadiazole-7-carbothioic-S-methyl-ester (BTH=Acibenzolar-S-methyl), capable of inducing SAR, has been tested against several pathogens (Kessmann *et al.*, 1994a; Ruess *et al.*, 1995; Oostendorp *et al.*, 1996). BTH ensured 90 per cent disease reduction on 17th day after its application as against only 46 per cent in case of metalaxyl. It is an effective inducer of resistance in tobacco not only against fungal pathogens, but also against viruses and bacteria (Tally *et al*., 1999). Brisset *et al*. (2000) reported that Acibenzolar-S-methyl protected Golden Delicious seedlings, scions and trees when applied before inoculation of *Erwinia amylovora*. The protection of apple seedlings was similar to the protection obtained with the standard for fire blight control (plantomycin) applied immediately before inoculation. The mean levels of control in scions in greenhouse and in trees in orchards were approximately 69 and 50 per cent, respectively. Resistance inducing effects of BTH product (BION®) have been demonstrated in plants against *Erysiphe graminis*, *Septoria* spp., *Pyricularia oryzae*, *Peronospora tabacina*,

Phytophthora spp., *Didymella bryonia*, (Ruess *et al.*, 1995; Kessmann *et al.*, 1996; Gorlach *et al.*, 1996) CMV-Y (cucumber mosaic virus) (Anfoka, 2000) and against *Erwinia amylovora* (Brisset *et al.*, 2000; Zeller and Zeller, 1998). Bishnoi and Payyavula (2003) tested the effect of two plant activators, Messenger® (a.i. Harpin protein) and Actigard™ (a.i. BTH) on three tomato *(Lycopersicon esculentum* Mill*)* cultivars (Mountain Pride, Floralina and Florida-47) and two canola (*Brassica napus* L.) cultivars, (Flint and 188-20B) and showed that in tomato, Messenger® and Actigard™ decreased early leaf blight (*Alternaria solani*) severity from 8-12% and increased tomato yield from 10-13 per cent in comparison to control, while in canola, the effect of both activators on crop maturity and severity of black leg (*Leptosphaeria maculans*) disease infection was non-significant. Similar results have been reported by Inbar *et al*. (1997) and Pervaiz *et al*. (2002) who found that application of Actigard™ resulted in significantly lower disease severity.

CHAPTER - 3

MATERIALS AND METHODS

CHAPTER – 3

MATERIALS AND METHODS

The present study was conducted during 2007-08-09 in the Division of Plant Pathology, SKUAST-K, Shalimar. The materials and methods relating to the objectives of the study are described under the following heads:

3.1 Status of the disease

The survey for the disease status was carried out in seven districts of Kashmir valley, namely, Bandipora, Baramulla, Budgam, Ganderbal, Kulgam, Pulwama and Shopian during late August (peak period of disease) in the 2007 and 2008. Random selection of three apple orchards from three sites per district was done for the purpose. Five trees selected randomly from these orchards were surveyed each year for recording disease incidence as well as intensity. The disease incidence was recorded by counting the number of diseased leaves among the total number of leaves on randomly selected four branches in four directions of each tree, using the following formula:

$$\text{Disease incidence (\%)} = \frac{\text{No. of infected leaves}}{\text{Total No. of leaves assessed}} \times 100$$

The disease intensity was recorded by visual observation using 0-5 scale (Filajdic and Sutton, 1991). Six categories were made on the basis of per cent leaf area diseased (Plate 1) as per the following key:

Category	Numerical value	Criteria
I	0	No symptoms
II	1	0 - 3% leaf area covered with lesions
III	2	4 - 6% leaf area covered with lesions
IV	3	7 - 12% leaf area covered with lesions
V	4	13 - 25% leaf area covered with lesions
VI	5	26 - 50% leaf area covered with lesions or chlorotic leaf with petiole infection

Per cent disease intensity (PDI) was calculated as per the formula:

$$\text{Per cent disease intensity} = \frac{\text{Sum of all the numerical ratings}}{\text{Number of leaves examined x maximum disease rating}} \times 100$$

Diseased apple leaves collected from surveyed sites were studied under stereobinocular microscope to record the mite (*Panonychus ulmi*) population.

3.2 Variability study

Variability studies were carried out under the following headings:

3.2.1 Collection of samples

During survey for disease assessment apple leaves from susceptible cultivar Red Delicious exhibiting typical symptoms of Alternaria leaf blotch

Plate 1: Scale (0-5) for assessment of Alternaria leaf blotch intensity

were collected from twenty one apple orchards across seven districts of Kashmir valley, *viz*., Bandipora, Baramulla, Budgam, Ganderbal, Kulgam, Pulwama and Shopian.

3.2.2 Isolation and purification of *Alternaria mali* isolates

For isolation of fungal pathogen, the diseased leaf area along with some healthy portion was cut into small bits with a sharp sterilized blade. These bits were surface sterilized in 0.1 per cent mercuric chloride ($HgCl_2$) for about 30 second followed by three washing in sterilized distilled water to remove the last traces of mercuric chloride (Johnston and Booth, 1983). After blotting dry with sterilized filter papers, these bits were transferred to sterilized potato dextrose agar medium (PDA) in sterilized petriplates. Three such bits were placed in each petriplates and incubated for seven days at $24\pm1^{o}C$.

The isolates were purified through single spore isolation technique (Johnston and Booth, 1983) by transferring germinating conidia to petriplates containing sterilized potato dextrose agar (PDA) medium. The pure cultures were maintained on PDA slants and stored at $4^{0}C$ in a refrigerator.

3.2.3 Pathogenicity

Pathogenicity of the isolates was carried out as under:

3.2.3.1 Preparation of inoculum

The isolates of *A. mali*, were multiplied on potato dextrose agar medium for 10 days at 24±1^{o}C in BOD incubator. After 10 days of incubation, spore suspension was prepared by flooding culture plate with sterilized distilled water, scraping with a sterilized razor blade, straining through a double layer of sterile cheesecloth into a 150 ml flask, and adjusting the spore concentration with haemocytometer to 4 x 10^5 conidia per millilitre. The prepared conidial suspensions were used for pathogenicity.

3.2.3.2 Pathogenicity

Pathogenicity of fungus was carried out through "Detached leaf technique" (Melouk and Banks, 1978) with slight modifications on Red Delicious cultivar. Healthy leaves were collected, washed thoroughly under running water, followed by three washings in sterilized water. After mild rinsing of leaf surface with sterilized water, the leaves were dried with a laboratory towel and placed in petriplates. Each petriplate was lined with a wet blotter paper on the bottom for maintaining high humidity. A set of three leaves, injured, uninjured and control was placed in each petriplate. The leaves were inoculated (outside the petriplates) with a conidial suspension (4 × 10^5 spores/ml) with an atomizer to the whole surface of the

injured and uninjured leaves, while as control was sprayed with the sterilized water. Leaves in the petriplates were incubated at 24±1^{o}C in BOD incubator with 12 hour dark and 12 hour light adjustment and monitored for symptom development. Re-isolations of the pathogen were carried out and compared with original inoculum to satisfy Koch's postulates.

3.2.4 Maintenance of *Alternaria mali* isolates

Maintenance of the pathogenic isolates was carried out by repeated sub culturing.

3.2.5 Cultural variability

Study for cultural variability among the twenty one isolates was made on the basis of:

3.2.5.1 Type and colour of colony

5 mm mycelial discs of 7 days old culture were transferred to the centre of PDA plates and incubated at 24±1^{o}C. Three replications were maintained for each isolate. Colony character *viz*: type, colour and margins were recorded after 10 days of growth. Reverse side of cultural plate of each fungal isolate was observed to record colour on underside of the plate.

3.2.5.2 Growth rate of fungus

To provide a uniform assessment of pathogen growth rates, isolates

were cultured on PDA in 90 mm Petri plates. A two step inoculation method was performed. *Alternaria mali* isolates from the stored collection were initially inoculated onto PDA and following suitable growth of each isolate, 5 mm fungal discs from the periphery of 7 days old fungal isolates were transferred to the centre of PDA Petri plates. The isolates were incubated at 24±1^{o}C. The colony diameter was recorded on 2nd, 4th, 6th, 8th and 10th day after incubation by taking two perpendicular measurements and their average calculated. The experiment was laid in CRD with three replications.

3.2.6 Morphological variability

Ten days old cultures of all the twenty one isolates grown on PDA were studied for any morphological variations. Temporary mounts were studied under compound microscope with respect to following characters:

Hyphae	:	Colour, width
Conidiophore	:	Septation, colour, size
Conidia	:	Septation, colour, size, shape

Measurements were taken with the help of software Magnus Pro. MLX Series, New Delhi, India. Fifty recordings per replication were made for the purpose.

3.2.7 Pathogenic variability

Pathogenic variability among the isolates was carried out through "Detached leaf technique" on susceptible Red Delicious cultivar. The experiment was carried out by employing the technique similar to that adopted for determining the pathogenicity test. Incubation period was recorded on the appearance of the first symptom, while as number of lesions and size of lesions were recorded 10 days after inoculation. The size of lesion was recorded by taking two perpendicular measurements and their average was calculated. The experiment was laid in CRD with three replications.

3.2.8 Data analysis of similarity of isolates on the basis of cultural, morphological and pathogenic characters

All the data in the Tables (Table 4-12) of cultural, morphological and pathogenic variability was digitalized into a two-discrete-character-matrix (0 and 1 for absence and presence of a particular character, respectively). The binary data of the table 4, 6 and 11 was generated on the basis of presence or absence of a particular character. The binary data of the Table 5, 7, 8, 9, 10 and 12 was generated on the basis of critical difference (CD). The data of all the characters was combined. Characters that could be scored univocally for presence and absence were included in analysis. Binary matrices were analyzed by NTYSYS-PC 2.1 and Similarity

coefficient was used to construct dendrogram using SHAN clustered programme, selecting the unweighted paired group method of arithmetic average (UPGMA) in NTYSYS-PC V 1.8 (Rohlf, 1993).

3.2.9 Molecular characterization

The genetic diversity of *Alternaria mali* isolates associated with Alternaria leaf blotch of apple was studied by employing random amplified polymorphic DNA (RAPD) technique (Williams *et al*., 1990).

3.2.9.1 Extraction of genomic DNA

Fungal mycelia were grown in conical flasks containing 100 ml potato dextrose broth. For this potato dextrose broth was inoculated with 2 plugs of 7 days old culture and incubated at 24±1^{o}C for 6-7 days. Mycelia were harvested by filtration through a double layered sterilized filter paper. The agar plugs were removed and resultant mycelia dried between two layers of filter paper in a laminar air flow cabinet and stored at −80^{0}C for further use. Total genomic DNA of each isolate was extracted using CTAB method (Murray and Thompson, 1980) with minor modifications. The dried mycelium was first ground to fine power in liquid nitrogen using pre-chilled pestle and mortar. The powder was immediately transferred to 1.5 ml micro-centrifuge tube containing 700 µl of extraction buffer (100 mM Tris HCl pH 8.0, 1.4 M NaCl, 20 mM EDTA pH 8.0, 2% CTAB, 1% PVP and

0.5% β-mercaptoethanol) and incubated for 1 hr in a shaking water bath at 65^{o}C. To each tube, equal volume (700 µl) of chilled chloroform: isoamyl alcohol (24:1) was added. The contents were mixed thoroughly and tubes spun at 10,000 rpm for 15 min in cooling centrifuge (Sigma Laboratory Centrifuges, Sartorius) at 4^{o}C. Aqueous phase was then transferred to new tube and equal volume of pre-chilled isopropanol added and kept at -20^{o}C overnight. The tubes were then centrifuged at 10,000 rpm for 10 minutes and supernatant decanted. The DNA pellets were washed thrice with 70 per cent ethanol, dried and dissolved in 200 µl of TE buffer (10 mM Tris HCl pH 8.0 and 1 mM EDTA, pH 8.0). RNase was added @ 10 µg/ml (MBI Fermentas Life Sciences) and emulsion was incubated for 2 hours at 37^{o}C. DNA was stored at -80^{o}C (Deep Freezer Co. Pvt. Ltd.) for further use.

3.2.9.2 Primer selection

Thirty 10 mer and twelve 20-mer oligonucleotide primers (Operon Technologies Inc., Alameda, CA, Sigma Genosys) were screened with 2 randomly selected isolates for polymorphism. The primers showing consistency in polymorphism among the isolates were selected for RAPD profiling of all the 21 *Alternaria mali* isolates. The base sequences of primers used for screening are listed in Table 14.

3.2.9.3 PCR conditions for RAPD

The PCR was carried out in 0.2 ml PCR tube with 25 µl reaction volume containing 1x buffer (20 mM Tris-HCl pH 8.0; 50 mM KCl), 1.5 mM $MgCl_2$, 2.0 µl dNTP mix (0.2 mM), 1 U of *Taq* DNA polymerase (5 U/µl), (Fermentas Life Sciences), 2 µl of DNA tempelate (20 ng), 5 pmols of primer and 17.3 µl of sterilized distilled water.

Reaction mixture was vortexed and centrifuged in a microfuge (Thermo Scientific, Thermo electron Corporation). Amplifications were performed using thermal cycler (Whatman Biometra, T Gradient, Goettingen, Germany) programmed for initial denaturation at 94^{o}C for 5 minutes followed by 40 cycles at 94^{o}C for 1 minute, 37/55 ^{o}C (37^{o}C for decamer and 55^{o}C for twenty-mer) for 1 minute, 72^{o}C for 2 minute and a final extension at 72^{o}C for 5 minutes using fastest ramp time between transitions.

3.2.9.4 Gel electrophoresis

The amplified PCR products were resolved by electrophoresis using 1.2 per cent (w/v) agarose gel in 1X Tris borate EDTA buffer (0.5 M Tris, 0.05 M boric acid, 1 mM EDTA, pH 8.0). The gels were stained with ethidium bromide (0.5 µg/ml). DNA ladders of 100 bp and Lambda

DNA/*EcoR1+Hind III* double digested marker (Fermentas Life Sciences) were used as markers. The gels were run at 80 V for one and a half hour using Consort Power Pack system (Consort EV 215).

3.2.9.5 Gel Images

The gel images were captured using a combination of Ultracam Digital Imaging (A 650 Canon Camera) and Electronic UV Transilluminator (Ultra.Lum.Inc, 1480 N, Claremont Boulevard, Claremont, CA).

3.2.9.6 Data analysis

Four most polymorphic and reproducible primers *viz.* P13, U2, U3 and U9 were used for RAPD profiling. The band pattern obtained from agarose gel electrophoresis was digitalized to a binary-matrix (0 and 1 for absence and presence of RAPD-bands, respectively). The data of all primers were combined. Bands that could be scored univocally for presence and absence were included in analysis. Binary matrices were analyzed by NTYSYS-PC 1.8 and Dice coefficient was used to construct dendrogram using SHAN clustered programme, selecting the unweighted paired group method of arithmetic average (UPGMA) in NTYSYS-PC V 1.8 (Rohlf, 1993). The dendrogram with best fit to similarity matrix based on cophenetic value (COPH) and matrix comparison (MXCOMP) was chosen.

3.3 Screening of cultivars

Potted plants of the commercially important cultivars of the apple *viz*: Akbar, American Apirouge, Firdous, Golden Delicious, Gulshan, Lal Ambri, Red Delicious, Red Gold, Shireen, Spartan, Star Crimson, Sunheri, and White Dotted Red were assessed for disease intensity during the year 2008 and 2009. Most virulent isolate (Am-1) recorded during the earlier experiment of pathogenic variability was used for screening these cultivars. Single spore isolate of Am-1 was allowed to grow for 7 days on PDA at 24 ± 1^{o}C. Spore suspension of 4×10^{5} conidia / ml was prepared adopting the method as discussed earlier in 'preparation of inoculum' and two to three drops of tween 20 were added to the suspension. Two year old plants were inoculated by spraying the spore suspension on both leaf surfaces to the drip point with an atomizer. Before inoculation celite (0.5%) was applied to the leaves of the plants with the help of a brush. Inoculation of plants was carried out under polyhouse conditions for maintenance of humidity. Forty eight hours after inoculation plants were moved to open under natural conditions. The experiment was laid in randomized block design with five replications for each cultivar. Plants were kept under observation for symptom expression and 10 days after the first symptom development, disease intensity was assessed by adopting scale as discussed earlier in

'status of the disease' chapter 3. The cultivars evaluated were arbitrarily categorized into five different reaction groups (Shahzad, 2003) on the basis of percent disease index (PDI) with some modifications as under:

Reaction category	Percent disease index (PDI)
Highly resistant	0.1 - 1.0
Resistant	1.1 - 5.0
Moderately resistant	5.1 - 10.0
Susceptible	10.1 - 20.0
Highly susceptible	> 20.0

3.4 Efficacy of systemic acquired resistance (SAR) activators for the management of disease

Seven SAR inducing compounds *viz*: 2, 6-dichloroisonicotinic acid (INA), benzothiadiazole S-methyl ester (BTH), β-aminobutyric acid (BABA), K_2HPO_4, K_3PO_4, $Ca(OH)_2$ and $CaCO_3$ were evaluated against the most virulent isolate (Am-1) of the pathogen on potted plants cv. Red Delicious under controlled conditions (Plate 1b). Test plants were divided in two sets each for chemical sprays 48 hrs before spore inoculation and 48 hrs after spore inoculation. Inoculum was provided to the test plants by spraying with spore suspension of seven days old culture. Spore inoculation

was made in the same manner as in the case of screening of cultivars. Before inoculation celite (0.5%) was applied to the leaves of the plants with the help of a brush. INA and BTH were tested at 50, 100 and 200 ppm concentration, BABA at 500, 1000 and 2000 ppm, K_2HPO_4, K_3PO_4, $Ca(OH)_2$ and $CaCO_3$ at 25, 50 and 100 mM. Penconazole at 300, 400 and 500 ppm concentration served as standard check whereas water was sprayed on check plants. Three replications were maintained for each treatment. All the chemicals were sprayed to the runoff with an atomizer. Data on disease intensity was recorded 15 days after spore inoculation as discussed earlier in 'status of the disease' chapter 3. The experiment was laid in CRD with three replications.

CHAPTER - 4

EXPERIMENTAL FINDINGS

CHAPTER – 4

EXPERIMENTAL FINDINGS

The experimental findings of the research work are presented under following heads:

4.1 Status of the disease

The disease survey was conducted in seven districts of Kashmir valley, namely, Bandipora, Baramulla, Budgam, Ganderbal, Kulgam, Pulwama and Shopian during late August in 2007 and 2008 and disease incidence and intensity was recorded.

4.1.1 Disease incidence

The overall mean disease incidence recorded in 2007 was 39.05 per cent as compared to 45.18 per cent in 2008 (Table 1). Alternaria leaf blotch disease was prevalent in all the seven districts surveyed with highest disease incidence of 51.94 percent in district Pulwama followed by 47.99 percent in district Ganderbal. In Budgam, Kulgam, Baramulla and Bandipora districts the disease incidence was 45.13, 44.32, 43.30 and 36.65 per cent, respectively. The lowest disease incidence of 25.49 per cent, irrespective of years was recorded in district Shopian. During 2007, the disease incidence varied from 22.85 per cent in district Shopian to 48.38 per cent in district

Pulwama and during 2008 it varied from 28.14 per cent in Shopian to 55.51 per cent in Pulwama.

The site-wise pooled data revealed the highest disease incidence of 68.74 per cent in Batwina of district Ganderbal (Table 1). This was followed by 55.48 per cent in Pinglena of district Pulwama, 52.88 per cent in Sehpora of district Kulgam and 51.56 per cent in Babber of district Pulwama. The least disease incidence of 19.33 per cent was recorded in Kachdoora (Distt. Shopian) followed by 22.18 per cent in Imam sahib (Shopian) and 32.32 per cent in Lar (Ganderbal). The disease incidence of sites ranged from 17.38 to 65.22 per cent in 2007 and 21.28 to 72.26 per cent in 2008. During both the years lowest disease incidence was recorded in Kachdoora (Shopian) and highest disease incidence in Batwina (Ganderbal). In district Bandipora, Aloosa recorded the highest disease incidence of 37.63 per cent while Bagh recorded the lowest of 34.97 per cent. In district Baramulla highest disease incidence of 46.11 per cent was recorded in Tarzoo and lowest of 40.63 per cent in Pattan. In district Budgam, Zaloosa recorded the highest disease incidence of 47.31 per cent while Chrawni recorded the lowest of 43.30 per cent. In district Ganderbal highest disease incidence of 68.74 per cent was recorded in Batwina and lowest of 32.32 per cent in Lar. In districts of Kulgam, Pulwama and

Table 1: Disease incidence of Alternaria leaf blotch of apple cv. Red Delicious in Kashmir valley

District	Site	Disease incidence (%)		
		2007	2008	Pooled Mean
Bandipora	Bagh	31.94	38.01	**34.97**
	Aloosa	34.86	40.41	**37.63**
	Asham	35.21	39.48	**37.34**
	Mean	**34.00**	**39.3**	36.65
Baramulla	Pattan	36.34	44.92	**40.63**
	Tarzoo	43.57	48.65	**46.11**
	Sopore	41.08	45.27	**43.17**
	Mean	**40.33**	**46.28**	43.30
Budgam	Chrawni	38.34	48.27	**43.30**
	Zaloosa	42.71	51.91	**47.31**
	Chadoora	43.14	46.44	**44.79**
	Mean	**41.39**	**48.87**	45.13
Ganderbal	Lar	30.29	34.36	**32.32**
	Batwina	65.22	72.26	**68.74**
	Gulab Bagh	40.36	45.50	**42.93**
	Mean	**45.29**	**50.70**	47.99
Kulgam	Vokia	39.20	45.17	**42.18**
	Amnoo	33.98	41.83	**37.90**
	Sehpora	50.29	55.47	**52.88**
	Mean	**41.15**	**47.49**	44.32
Pulwama	Tral	45.97	51.61	**48.79**
	Babber	47.62	55.51	**51.56**
	Pinglena	51.56	59.41	**55.48**
	Mean	**48.38**	**55.51**	51.94
Shopian	Memender	31.25	38.73	**34.99**
	Kachdoora	17.38	21.28	**19.33**
	Imam Sahib	19.94	24.43	**22.18**
	Mean	**22.85**	**28.14**	25.49
Overall Mean		**39.05**	**45.18**	**42.11**

Shopian highest disease incidence was recorded in Sehpora (52.88%), Pinglena (55.48%) and Memender (31.25%), while as lowest disease incidence was recorded in Amnoo (37.90%), Tral (48.79%) and Kachdoora (19.33%) respectively.

4.1.2 Disease intensity

The data presented in Table 2 revealed that overall disease intensity of 24.38 per cent recorded in 2008 was higher than 19.00 per cent recorded in 2007. The pooled data showed that highest disease intensity of 26.34 per cent was recorded in Pulwama followed by 25.48 per cent in Ganderbal. The lowest disease intensity of 12.52 per cent, irrespective of years was recorded in district Shopian. The disease intensity of 23.58, 23.23, 21.68 and 19.00 per cent was recorded in Budgam, Kulgam, Baramulla and Bandipora, respectively. The disease intensity in districts ranged from 11.36 to 22.30 per cent in 2007 and 13.69 to 30.36 per cent in 2008.

Disease intensity in different sites varied from 8.33 to 30.32 per cent in 2007 and 10.10 to 36.91 per cent in 2008 (Table 2). The pooled data revealed that the disease intensity varied from 9.21 to 33.62 per cent with the highest in Batwina (Ganderbal) followed by 27.65 per cent in Pinglena (Pulwama), 26.90 per cent in Tarzoo (Baramulla), 26.81 per cent in Babber (Pulwama), 26.11 per cent in Zaloosa (Budgam) and 25.84 per cent in

Gulab bagh (Ganderbal). The lowest disease intensity of 9.21 per cent was recorded in Kachdoora (Shopian) while as it was 12.97 per cent in Imam sahib (Shopian), 15.40 per cent in Memender (Shopian) and 16.36 per cent in Aloosa (Bandipora). In Bandipora highest disease intensity of 22.31 per cent was recorded in Asham followed by 18.32 per cent in Bagh and 16.36 per cent in Aloosa. In Baramulla, Tarzoo recorded the disease intensity of 26.90 per cent followed by 20.50 per cent in Pattan and 17.63 per cent in Sopore. Out of the three sites surveyed in Budgam, Zaloosa recorded the highest disease intensity of 26.11 per cent, while as Chadoora and Chrawni recorded 22.56 and 22.09 per cent, respectively. In Ganderbal highest disease intensity of 33.62 per cent was recorded in Batwina and the lowest of 16.98 per cent in Lar. Gulab bagh recorded the disease intensity of 25.84 per cent. In Kulgam, Amnoo recorded the highest disease intensity of 24.31 per cent followed by 22.89 per cent in Vokai and 22.49 per cent in Sehpora. In Pulwama highest disease intensity was recorded in Pinglena (27.65%), followed by Babber (26.81%) and Tral (24.54%). In Shopian highest disease intensity of 15.40 per cent was recorded in Memender followed by 12.97 per cent in Imam sahib and 9.21 per cent in Kachdoora. Comparatively higher mite (*Panonychus ulmi*) population was observed at sties with higher disease intensities.

Table 2: Disease intensity of Alternaria leaf blotch of apple cv. Red Delicious in Kashmir valley

District	Site	Disease intensity (%)		
		2007	2008	Pooled Mean
Bandipora	Bagh	15.72	20.93	**18.32**
	Aloosa	13.35	19.37	**16.36**
	Asham	19.98	24.65	**22.31**
	Mean	**16.35**	**21.65**	19.00
Baramulla	Pattan	18.36	22.65	**20.50**
	Tarzoo	23.69	30.12	**26.90**
	Sopore	15.28	19.98	**17.63**
	Mean	**19.11**	**24.25**	21.68
Budgam	Chrawni	18.29	25.89	**22.09**
	Zaloosa	22.80	29.42	**26.11**
	Chadoora	20.62	24.50	**22.56**
	Mean	**20.57**	**26.60**	23.58
Ganderbal	Lar	15.17	18.79	**16.98**
	Batwina	30.32	36.91	**33.62**
	Gulab Bagh	20.51	31.18	**25.84**
	Mean	**22.00**	**28.96**	25.48
Kulgam	Vokia	20.59	25.19	**22.89**
	Amnoo	24.62	24.01	**24.31**
	Sehpora	18.87	26.12	**22.49**
	Mean	**21.36**	**25.10**	23.23
Pulwama	Tral	21.53	27.55	**24.54**
	Babber	24.52	29.11	**26.81**
	Pinglena	20.87	34.44	**27.65**
	Mean	**22.30**	**30.36**	26.34
Shopian	Memender	14.49	16.32	**15.40**
	Kachdoora	8.33	10.10	**9.21**
	Imam Sahib	11.27	14.67	**12.97**
	Mean	**11.36**	**13.69**	12.52
Overall Mean		**19.00**	**24.38**	**21.68**

4.2 Variability study

Variability amongst the pathogenic isolates was recorded with respect to cultural, morphological, pathogenic and molecular characters.

4.2.1 Isolation and purification of *Alternaria mali* isolates

A total number of 21 isolates of *Alternaria mali* were obtained from 21 different sites of the seven districts surveyed. Isolations were made from diseased leaf tissue of apple cultivar "Red Delicious". After 48 hours incubation of diseased leaf tissue on PDA medium at 24±1^{o}C, hyaline to light olivacious mycelial growth was observed. The cultural and morphological characters of the isolated fungus closely resembled to those described by Roberts (1924).

4.2.2 Pathogenicity and maintenance of *Alternaria mali* isolates

Pathogenicity tests of isolated fungi on injured and uninjured detached leaves of Red Delicious apple cultivar revealed the development of typical disease symptoms on injured leaves within 2-7 days of inoculation. However, no such symptoms developed on uninjured leaves even after 15 days of inoculation. Reisolation from these infected leaves yielded the fungi similar to inoculated ones thus fulfilling the Koch's postulates.

The pathogenic cultures were maintained by sub-culturing at monthly intervals and stored in a refrigerator for further studies. Thus twenty one isolates of *Alternaria mali* obtained from different sites satisfied Koch's postulates and were named Am-1 to Am-21 (Table 3).

4.2.3 Cultural variability

Isolates of *Alternaria mali* differed with respect to their cultural characteristics. The characters *viz*. type and color of colony, growth rate of fungus and pigmentation were recorded.

4.2.3.1 Type and color of colony

The *A. mali* isolates grown on PDA showed variation in their colony characteristics (Table 4). Colony colour varied from light to dark olivacious with greenish or brownish tinge. Mostly the colonies had velvety or cottony mycelial growth with regular to irregular margin (Plate 2-3). Colonies developed were either cottony or velvety with slight variations. The velvetty growth was observed in thirteen isolates *viz*. Am-1, Am-2, Am-4, Am-5, Am-8, Am-11, Am-12, Am-14, Am-15, Am-16, Am-17, Am-18, Am-20 and cottony type of growth in eight isolates *viz*. Am-3, Am-6, Am-7, Am-9, Am-10, Am-13, Am-19, Am-21. Among the velvety type of colonies variations recorded were as follows: Appressed growth was observed in Am-5, Am-11, Am-12, Am-14, Am-15, Am-16, Am-17, Am-

Table 3: Origin and naming of *Alternaria mali* isolates

Isolate name	Location	District
Am-1	Bagh	Bandipora
Am-2	Aloosa	
Am-3	Asham	
Am-4	Pattan	Baramulla
Am-5	Tarzoo	
Am-6	Sopore	
Am-7	Chrawni	Budgam
Am-8	Zaloosa	
Am-9	Chadoora	
Am-10	Lar	Ganderbal
Am-11	Batwina	
Am-12	Gulab Bagh	
Am-13	Vokia	Kulgam
Am-14	Amnoo	
Am-15	Sehpora	
Am-16	Tral	Pulwama
Am-17	Babber	
Am-18	Pinglena	
Am-19	Memender	Shopian
Am-20	Kachdoora	
Am-21	Imam Sahib	

Table 4: Cultural variability in *Alternaria mali* isolates

Isolate	Colony		
	Type	**Colour**	**Margin**
Am-1	Velvety, appressed, sparse cottony centre	Dark olivaceous green	Irregular, light Olivaceous green with white rim
Am-2	Velvety, cottony central growth	Olivaceous	Slightly irregular, Olivaceous green
Am-3	Cottony, slightly furrowed with appressed centre	Dark olivaceous, with dark centre	Regular, cottony brownish margin with whitish rim
Am-4	Velvety, with cottony central growth	Olivaceous green	Slightly irregular, Olivaceous green
Am-5	Velvety, Appressed	Light olivaceous green	Regular, light brownish margin with white rim
Am-6	Cottony, with slighty appressed margins	Olivaceous green with grayish surface	Regular, brownish with white rim
Am-7	Cottony, appressed centre	Light olivaceous green	Regular, cottony with white rim
Am-8	Velvety, slightly furrowed with appressed centre	Olivaceous with brownish centre and grayish surface	Irregular, appressed, Olivaceous green
Am-9	Cottony	Olivaceous green with grayish surface at centre	Regular, brownish with white rim
Am-10	Cottony	Light olivaceous green	Regular, with concentric rings of white and green, rim white
Am-11	Velvety, appressed	Olivaceous green with white cottony patches	Regular, green with grayish rim
Am-12	Velvety, Appressed	Olivaceous	Irregular, appressed, Olivaceous with white rim
Am-13	Cottony, sub-aerial	Dark green with greyish surface	Regular, dirty white margin
Am-14	Velvety, appressed	Olivaceous green	Slightly irregular, light green with grayish rim
Am-15	Velvety, appressed	Olivaceous	Slightly irregular, brownish with dirty white rim
Am-16	Velvety, appressed	Dark olivaceous green	Irregular, light Olivaceous with grayish rim
Am-17	Velvety, appressed	Dark olivaceous green	Slightly irregular, light Olivaceous with grayish rim
Am-18	Velvety, appressed	Olivaceous green	Slightly irregular, light green with grayish rim
Am-19	Cottony, slightly furrowed	Olivaceous	Regular, cottony olivaceous with greyish rim
Am-20	Velvety, appressed	Olivaceous green	Regular, appressed, Olivaceous green
Am-21	Cottony, sub-aerial	Greenish with grayish surface	Regular, appressed green with white rim

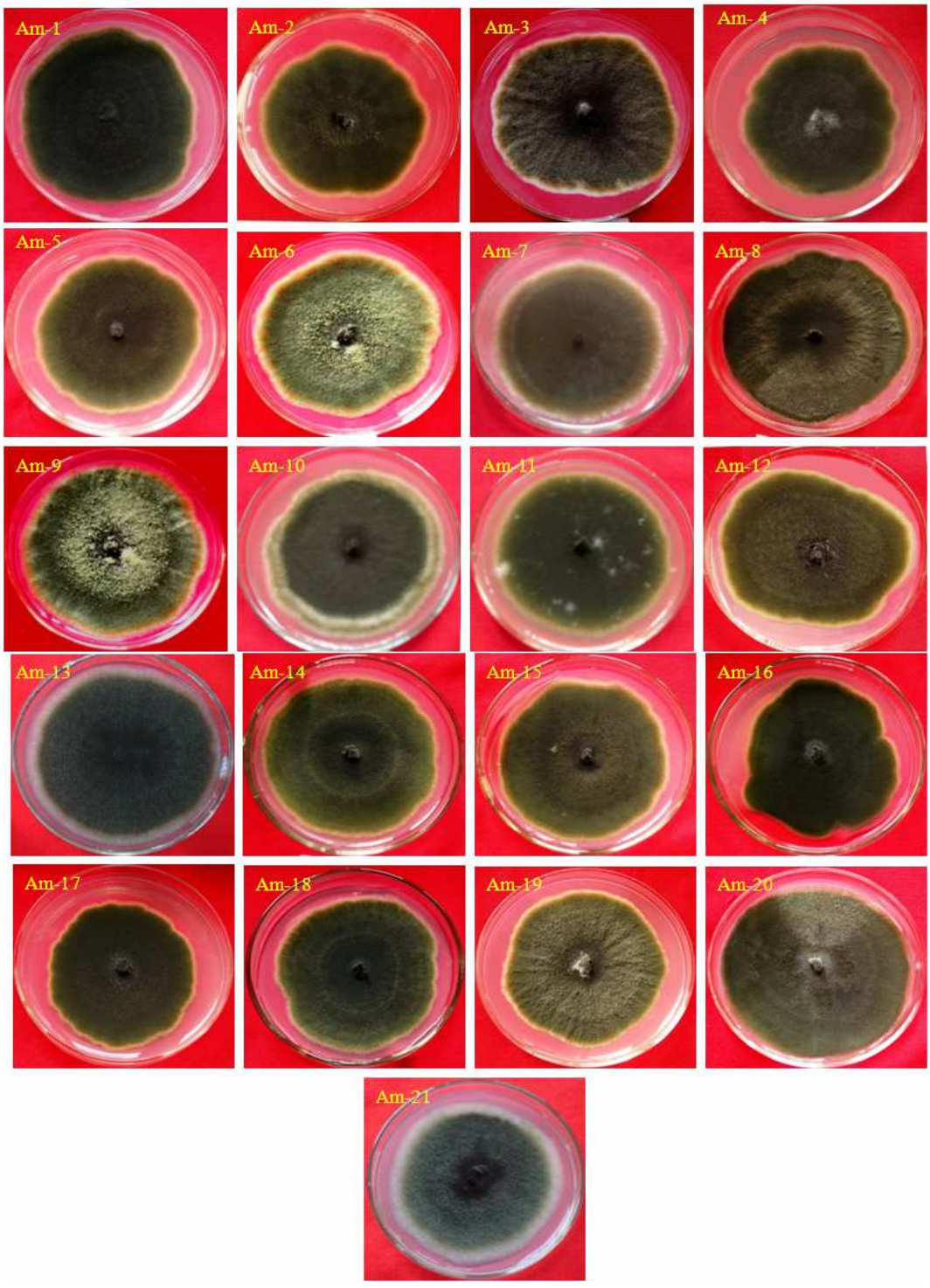


Plate 2: Cultural variability in *Alternaria mali* isolates

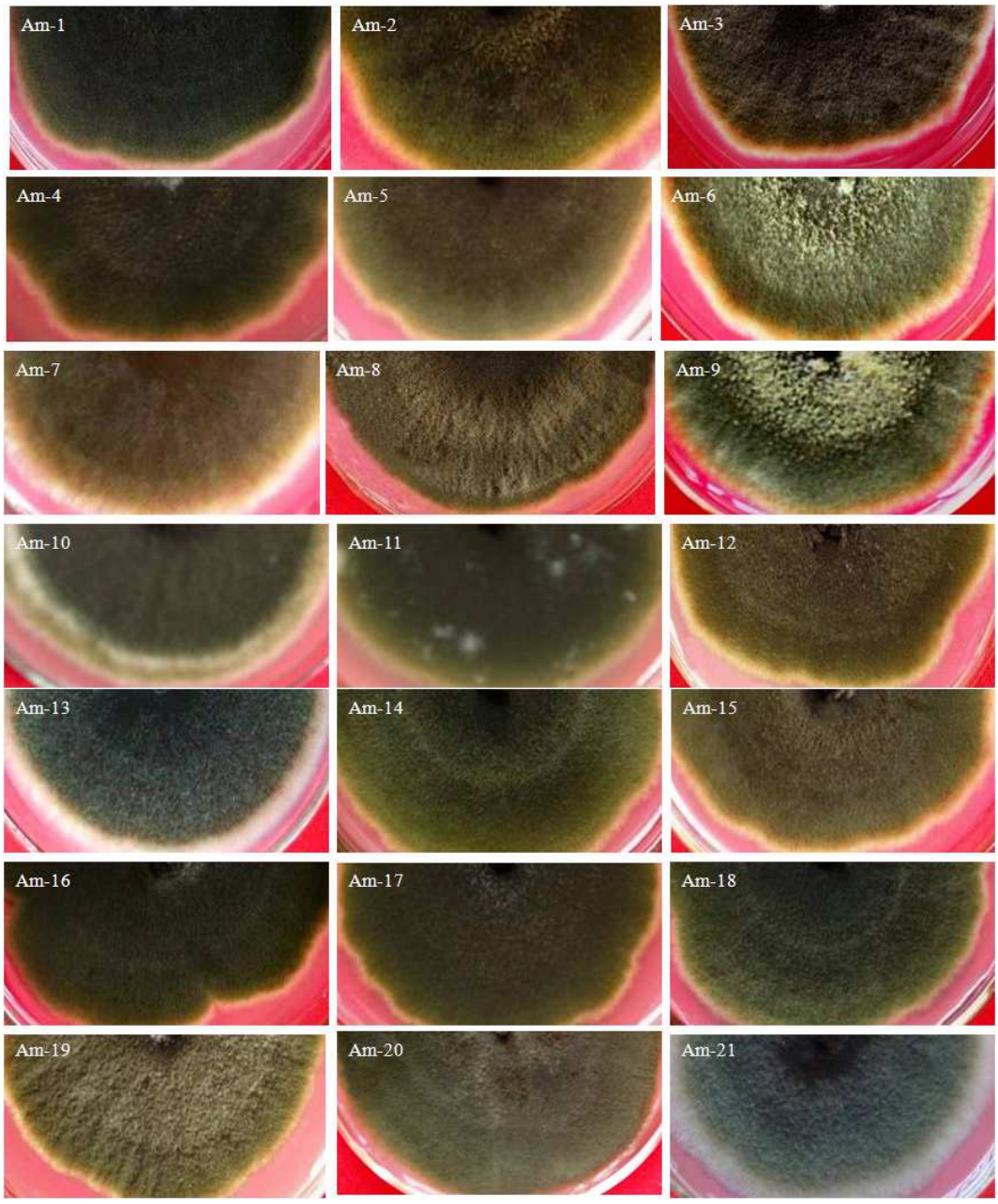


Plate 3: A close view of Cultural variability in *Alternaria mali* isolates

18 and Am-20. Am-1 also with appressed growth developed sparse cottony centre. Am-2 and Am-4 though velvety had cottony central growth. In Am-8 furrows and central appressed growth was recorded. Among the cottony type of colonies furrows developed in Am-3 and Am-19, appressed centre was recorded in Am-3 and Am-7 and sub-aerial mycelial growth in Am-13 and Am-21. The colony of Am-6 was with slightly appressed margins.

Isolates studied also varied in colony colours (Plate 2-3, Table 4). Dark olivacious green colonies were observed in three isolates *viz*. Am-1, Am-16 and Am-17. Four isolates *viz*. Am-2, Am-12, Am-15, Am-19 were olivacious, one isolate Am-3 was dark olivacious but with dark centre, four isolates *viz*. Am-4, Am-14, Am-18, Am-20 were olivacious green and three isolates *viz*. Am-5, Am-7, Am-10 were light olive green. Six colonies depicted some additional colour variations. Greyish superficial mycelial growth was observed on olivacious green colony of Am-6, dark green colony of Am-13 and greenish colony of Am-21. Greyish patches were observed on olivacious green colony of Am-11 while as Am-9 had grayish growth only in the centre of olivacious green colony. Am-8 was olivacious with grayish surface and brownish centre.

The colony margins varied from regular to irregular (Plate 2, Table 4). Regular margins were observed in eleven isolates viz. Am-3, Am-5,

Am-6, Am-7, Am-9, Am-10, Am-11, Am-13, Am-19, Am-20, Am-21. Four isolates (Am-1, Am-8, Am-12, Am-16) had irregular margins and remaining six (Am-2, Am-4, Am-14, Am-15, Am-17, Am-18) were with slightly irregular margins. Margins were light olivacious in Am-16 and Am-17, olivacious in Am-12 and Am-19, light olive green in Am-1, olive green in Am-2, Am-4, Am-8 and Am-20, light green in Am-14 and Am-18, green in Am-11 and Am-21. Am-5 developed light brown margins and brown was observed in Am-3, Am-6, Am-9 and Am-15. Dirty white margins were observed in Am-7 and Am-13 and margins with concentric rings of white and green in Am-10. In some cases margins were followed by white or grey rim.

4.2.3.2 Growth rate of fungus

All the isolates tested depicted variation in the growth rate (growth/day) (Table 5). Am-13 with mean growth rate of 8.21 mm was fastest but statistically at par with Am-4 (8.08 mm) and Am-6 (8.05 mm). These were followed by Am-9, Am-10 and Am-21 with mean growth rate of 7.99, 7.83 and 7.82 mm, respectively. Least growth rate of 5.86 mm was recorded in Am-5 which was statistically at par with 6.04 mm in Am-8.

Isolates also depicted periodic change in their growth rates. After 2, 4, 6 and 8 days of inoculation highest growth rate of 5.25, 10.75, 13.00 and

Table 5: Variability in the growth rate of *Alternaria mali* isolates

Isolate ⬇ / Days➔	Growth (mm/day) at different intervals					
	1-2	**3-4**	**5-6**	**7-8**	**9-10**	**Mean**
Am-1	2.25	6.91	9.91	13.41	4.66	**7.42**
Am-2	3.50	7.75	10.08	13.66	0.66	**7.13**
Am-3	5.25	8.65	11.75	12.40	0.75	**7.76**
Am-4	3.25	9.33	10.16	16.41	1.25	**8.08**
Am-5	2.66	5.58	8.66	9.75	2.66	**5.86**
Am-6	3.00	6.30	10.75	16.70	3.50	**8.05**
Am-7	1.25	8.50	13.00	14.30	0.83	**7.57**
Am-8	2.16	6.10	7.40	10.50	4.08	**6.04**
Am-9	2.75	7.75	10.90	13.00	5.58	**7.99**
Am-10	4.00	8.50	9.75	11.66	5.25	**7.83**
Am-11	1.16	8.91	10.58	14.25	0.16	**7.01**
Am-12	4.16	8.66	10.83	12.91	1.33	**7.57**
Am-13	1.75	10.75	12.66	13.58	2.33	**8.21**
Am-14	4.58	5.50	6.50	18.35	2.50	**7.48**
Am-15	3.66	6.25	10.25	12.33	2.75	**7.04**
Am-16	3.75	5.00	9.25	14.30	1.58	**6.77**
Am-17	2.25	7.58	10.33	13.41	3.41	**7.39**
Am-18	1.08	5.95	9.50	13.65	2.16	**6.46**
Am-19	3.33	4.16	8.75	10.75	5.66	**6.53**
Am-20	2.91	6.66	12.25	14.75	2.16	**7.74**
Am-21	2.66	9.33	10.91	12.91	3.33	**7.82**
Mean	**2.92**	**7.33**	**10.19**	**13.47**	**2.69**	7.32
$CD_{(P = 0.05)}$						
Days : 0.10; Isolate : 0.21; Isolate x Days : 0.48						

18.35 mm were recorded in Am-3, Am-13, Am-7 and Am-14 respectively. However after 10 days of inoculation highest growth rate of 5.58 mm was recorded in Am-9. The least growth rate recorded at 2, 4, 6, 8 and 10 days after inoculation was 1.08 mm in Am-18, 4.16 mm in Am-19, 6.50 mm in Am-14, 9.75 mm in Am-5 and 0.16 mm in Am-11, respectively. The maximum growth rate recorded in Am-3 two days after inoculation was significantly higher than all other isolates. The minimum growth rate of 1.08 mm recorded after two days of inoculation in Am-18 was statistically at par with 1.16 mm in Am-11 and 1.25 mm in Am-7, however significantly lower than all other isolates. After 4 days of inoculation the highest growth rate of 10.75 mm (Am-13) and lowest of 4.16 mm (Am-19) were significantly different from all other isolates. The highest growth rate of 13.00 mm recorded six days after inoculation in Am-7 was statistically at par with 12.66 mm in Am-13, but was significantly higher than all other isolates, while as the growth rate of 6.50 mm recorded in Am-14 was significantly lowest of all the isolates. The highest and lowest growth rate of 18.35 and 9.75 mm recorded eight days after inoculation in Am-14 and Am-5, respectively were significantly different from all other isolates. After 10 days of inoculation highest growth rate of 5.58 mm in Am-9 was statistically at par with 5.66 mm in Am-19 and 5.25 mm in Am-10, however significantly higher than other isolates. The lowest growth rate of

0.16 mm ten days after inoculation recorded in Am-11 was significantly lower than other isolates.

The overall growth rate was lowest (2.92 mm) after 2 days of inoculation and highest (13.47 mm) after 8 days of inoculation however, there was an increasing trend of growth rate upto 8^{th} day but decreased afterwards (Fig. 1).

4.2.3.3 Colour on underside of the plate

All the isolates impregnated the media with a colour mostly grey to brown with some variations which were clearly visible from the underside of plates (Plate 4, Table 6). Three isolates (Am-1, Am-4 and Am-8) imparted dark grey colour with light grey margin, three isolates (Am-2, Am-11 and Am-15) were brown with light grey margin and two (Am-3 and Am-10) were smoky grey to dark grey. Am-5, Am-9, Am-16 and Am-20 produced smoky grey colour and bluish brown with light grey margin was observed in Am-7 and Am-13. Two isolates (Am-12 and Am-14) were light grey with brown centre and two (Am-18 and Am-21) were light grey with dark grey centre. One isolate (Am-6) produced grey with light grey margin and Am-17 was light brown with light grey margin and dark brown centre, while the colour produced by Am-19 was greyish with light grey margin and brown centre.

Table 6: Media impregmentation by various isolates of *Alternaria mali*

Isolate	colour on underside of the plate
Am-1	Dark grey with light grey margin
Am-2	Brown with light grey margin
Am-3	Smoky grey to dark grey
Am-4	Dark grey with light grey margin
Am-5	Smoky grey
Am-6	Grey with light grey margin
Am-7	Bluish brown with light grey margin
Am-8	Dark grey with light grey margin
Am-9	Smoky grey
Am-10	Smoky grey to dark grey
Am-11	Brown with light grey margin
Am-12	Light grey with brown centre
Am-13	Bluish brown with light grey margin
Am-14	Light grey with brown centre
Am-15	Brown with light grey margin
Am-16	Smoky grey
Am-17	Light brown with light grey margin and dark brown centre
Am-18	Light grey with dark grey centre
Am-19	Greyish with light grey margin and brown centre
Am-20	Smoky grey
Am-21	Light grey with dark grey centre

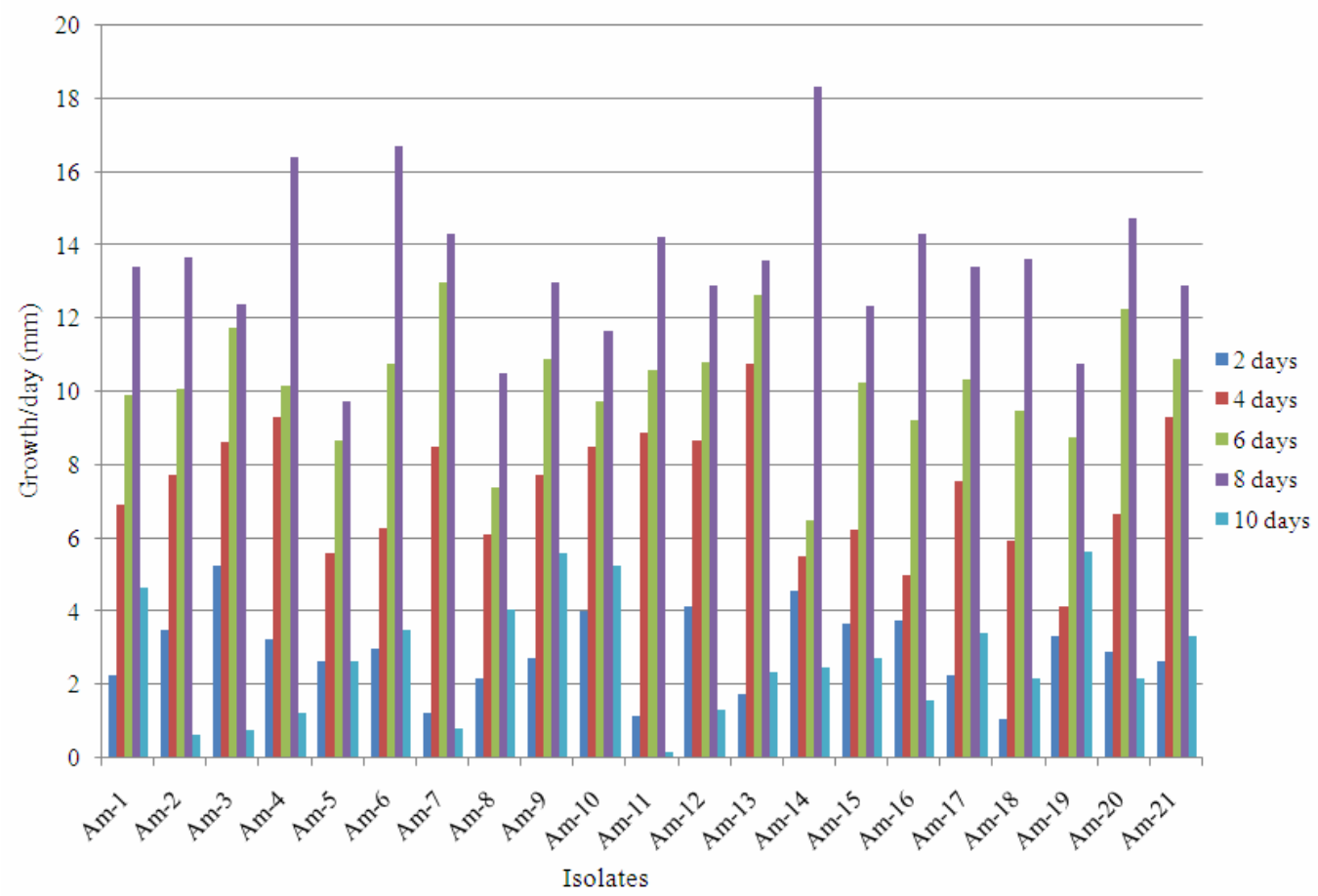


Fig. 1: Variability in the growth rate of *Alternaria mali* isolates

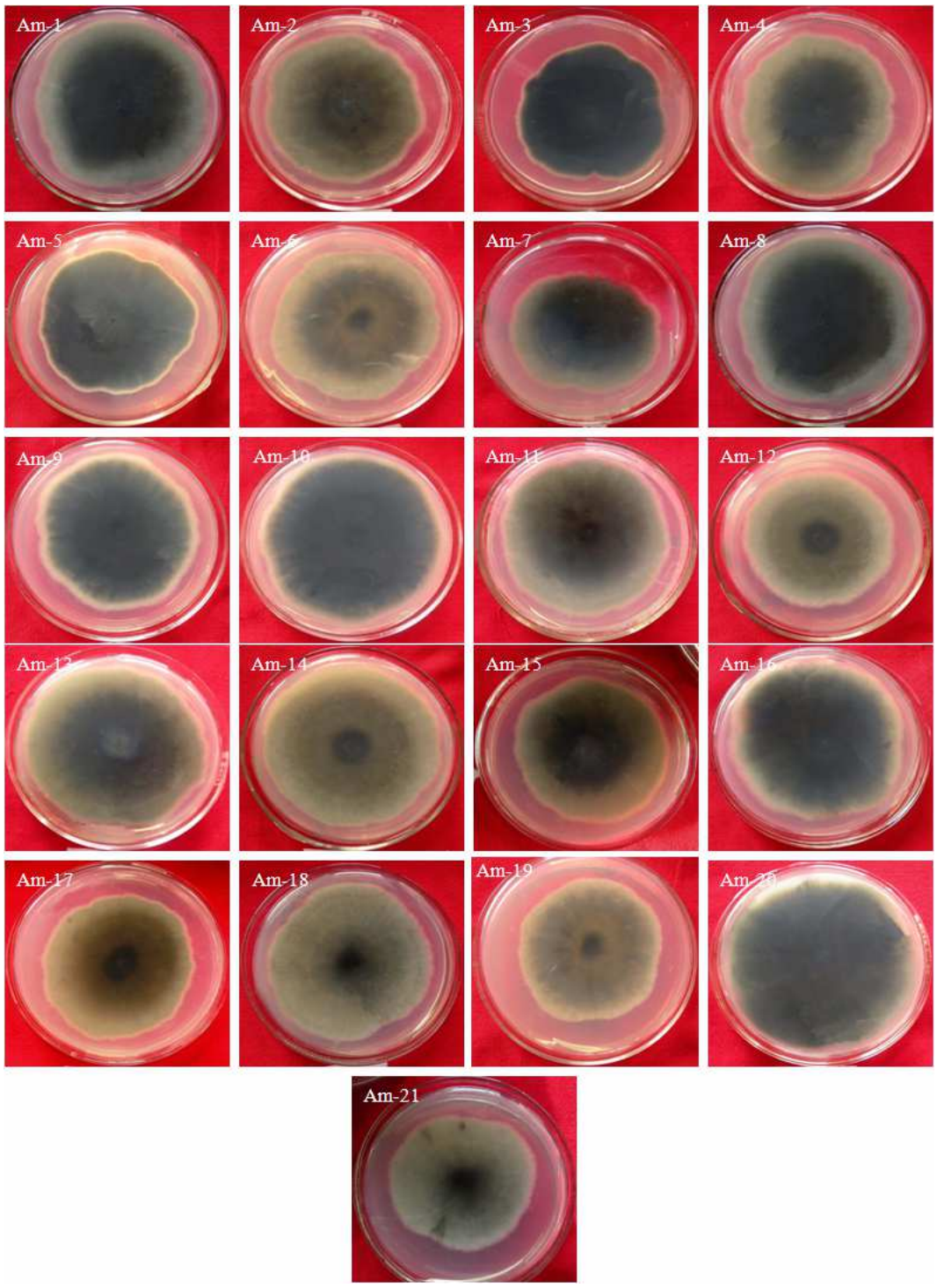


Plate 4: Variability of *Alternaria mali* isolates in colour development on underside of the plate

4.2.4 Morphological variability

Variations were observed amongst the isolates with respect to morphological characters like hyphal width and colour, conidiophore septation, colour and size, and conidial septation, colour, size and shape.

4.2.4.1 Hyphal width

All the isolates had hyphal strands of varied dimensions with mean hyphal width ranging from 3.28 to 4.28 µm (Table 7). Maximum hyphal width was in Am-4 and minimum in Am-21. However, Am-4 was at par with Am-3 (4.24 µm), Am-1 (4.21 µm), Am-5 (4.20 µm) Am-11 (4.19 µm), Am-2 (4.14 µm), Am-9 (4.14 µm), Am-18 (4.12 µm), Am-10 (4.09 µm), Am-16 (4.09 µm), Am-15 (4.02 µm), Am-7 (3.99 µm), Am-17 (3.99 µm), Am-6 (3.93 µm) and Am-8 (3.89 µm). Similarly Am-21 was at par with Am-19 (3.35 µm), Am-20 (3.47 µm), Am-14 (3.51 µm), Am-12 (3.54 µm) and Am-13 (3.58 µm). Range data revealed the least hyphal width of 2.19 µm in Am-21 and a maximum of 5.37 µm in Am-16.

4.2.4.2 Conidiophore variability

Morphological studies of conidiophores revealed variations in the size and septation amongst the isolates (Table 8). Conidiophore length ranged from 33.01 to 62.58 µm with maximum in Am-9 which was at par with Am-10 (60.75 µm) but significantly higher than all the other isolates.

Table 7: Variability in the hyphal width of *Alternaria mali* isolates

Isolate	Hyphal width (µm)	
	Range	Mean*
Am-1	3.75-5.20	4.21
Am-2	2.94-4.74	4.14
Am-3	3.81-5.25	4.24
Am-4	3.80-5.32	4.28
Am-5	3.92-4.95	4.20
Am-6	3.25-4.15	3.93
Am-7	3.45-4.20	3.99
Am-8	2.55-4.86	3.89
Am-9	3.65-4.51	4.14
Am-10	3.35-4.16	4.09
Am-11	3.71-4.99	4.19
Am-12	2.74-4.15	3.54
Am-13	2.94-4.12	3.58
Am-14	3.31-3.90	3.51
Am-15	2.78-5.06	4.02
Am-16	3.31-5.37	4.09
Am-17	3.45-4.23	3.99
Am-18	3.55-4.28	4.12
Am-19	2.91-3.87	3.35
Am-20	2.83-4.08	3.47
Am-21	2.19-4.14	3.28
$CD_{(P = 0.05)}$		0.58

* Average of 150 observations

Table 8 : Variability in the conidiophore of *Alternaria mali* isolates

Isolate	Conidiophore					
	Length (µm)		Breadth (µm)		Septation (No.)	
	Range	Mean*	Range	Mean*	Range	Mean*
Am-1	33.54-55.38	47.26	3.48 - 5.09	4.65	0-7	4.44
Am-2	42.35-59.69	55.66	3.85 - 5.47	4.40	1-5	3.08
Am-3	32.44-49.35	42.91	3.35 - 5.53	4.70	1-6	4.43
Am-4	36.57-54.61	47.76	3.47 - 5.40	4.60	1-9	4.54
Am-5	40.28-56.29	50.59	3.36 - 4.95	4.62	0-8	5.90
Am-6	41.32-58.65	53.72	4.06 - 5.62	4.57	1-5	4.06
Am-7	48.25-61.25	57.13	3.81 - 5.47	4.67	1-6	4.35
Am-8	34.55-50.43	43.61	4.27 - 5.05	4.60	1-7	4.78
Am-9	49.27-68.18	62.58	3.38 - 5.15	4.42	0-5	3.46
Am-10	47.49-65.41	60.75	3.67 - 5.24	4.45	1-4	3.69
Am-11	47.31-61.47	55.81	3.36 - 4.95	4.38	1-6	4.74
Am-12	33.29-53.68	42.59	3.49 - 5.08	4.54	1-7	5.14
Am-13	36.29-55.22	45.59	3.65 - 5.24	4.57	1-6	5.23
Am-14	27.28-44.95	33.01	4.20 - 5.34	4.46	0-4	3.28
Am-15	30.25-50.26	38.18	3.68 - 5.06	4.39	2-9	5.64
Am-16	28.01-49.88	37.50	3.36 - 4.78	4.03	1-4	3.09
Am-17	26.99-52.91	41.06	2.19 - 4.70	3.86	2-6	4.27
Am-18	28.71-44.21	34.03	3.21 - 4.68	4.00	1-5	4.19
Am-19	31.32-48.76	39.21	2.55 - 4.63	3.38	0-6	4.21
Am-20	28.21-48.58	36.92	2.79 - 4.41	3.30	1-8	3.94
Am-21	28.15-55.45	39.89	2.35 - 3.59	3.45	0-6	4.32
$CD_{(P = 0.05)}$		4.44		0.59		0.77

* Average of 150 observations

Minimum conidiophore length (33.01 µm) was recorded in Am-14 which was at par with Am-18 (34.03 µm) and Am-20 (36.92 µm) but significantly lower than all the other isolates. Range data of the conidiophore length revealed the maximum of 68.18 µm in Am-9 and the minimum of 26.99 µm in Am-17.

Similarly mean conidiophore breadth varied from 3.30 to 4.70 µm amongst the isolates (Table 8). The least was in Am-20 which was at par with Am-19 (3.38 µm), Am-21 (3.45 µm) and Am-17 (3.86 µm). The maximum was in Am-3 which was at par with fourteen other isolates *viz*: Am-7 (4.67 µm), Am-1 (4.65 µm), Am-5 (4.62 µm), Am-4 and Am-8 (4.60 µm), Am-6 and Am-13 (4.57 µm), Am-12 (4.54 µm), Am-14 (4.46 µm), Am-10 (4.45 µm), Am-9 (4.42 µm), Am-2 (4.40 µm), Am-15 (4.39 µm) and Am-11(4.38 µm). Range data of the conidiophore breadth showed maximum breadth (5.62 µm) in Am-6 and the minimum (2.19 µm) in Am-17.

Studies on conidiophore septation revealed that Am-5 had maximum mean septation (5.90) which was at par with Am-15 (5.64), Am-13 (5.23) and Am-12 (5.14). The least mean septation was in Am-2 (3.08) but was at par with Am-16 (3.09), Am-14 (3.28), Am-9 (3.46) and Am-10 (3.69). Conidiophore septation among the isolates ranged from 0 to 9 (Plate 5).

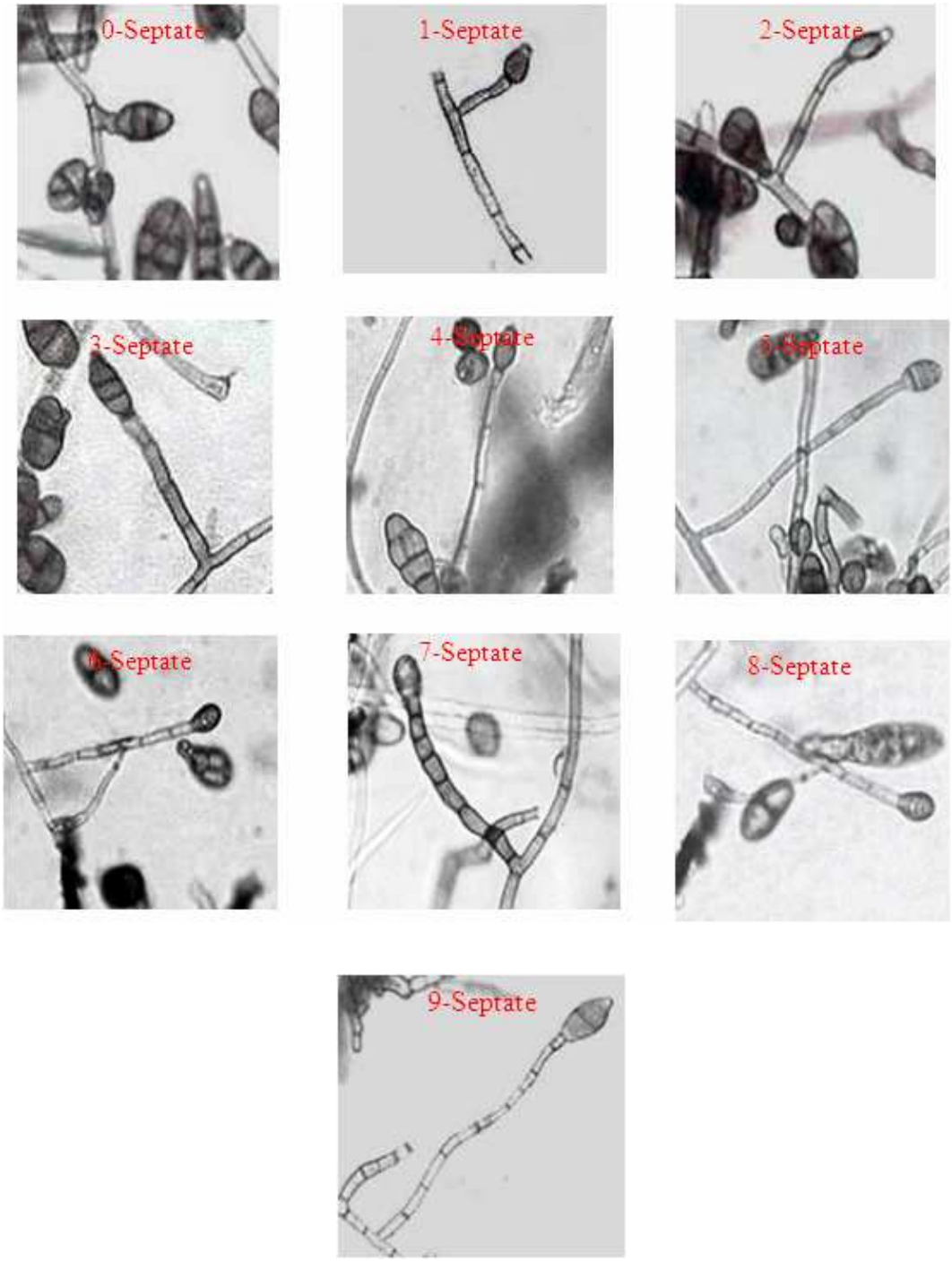


Plate 5: Conidiophore septation of *Alternaria mali*

Aseptate conidiophores were observed in Am-1, Am-5, Am-9, Am-14, Am-19 and Am-21. The highest number of septa (9) was recorded in Am-4.

4.2.4.3 Conidial septation

Isolates varied significantly in their transverse and longitudinal septation (Table 9) with transverse septation ranging from 1 to 7 and longitudinal from 0 to 4 (Plate 6). The highest mean number of transverse septa (2.95) was observed in Am-6 which was at par with Am13 (2.92), Am-18 (2.78), Am-21 (2.77) and Am-5 (2.75). The lowest mean number of transverse septa (1.76) was in Am-8 but was at par with 2.00 in Am-15. The highest mean number of longitudinal septa (1.12) was in Am-1 and was at par with 1.04 in Am-6, while the lowest (0.22) was in Am-14 which was at par with 0.33 in Am-8. Conidia with one transverse septum were observed in all the isolates, while the maximum of 7 was in Am-18. Conidia with no longitudinal septa were observed in all the isolates, while the maximum of 4 was in Am-3, Am-5, Am-9 and Am-12.

4.2.4.4 Conidial size

Average conidial size of isolates varied from 21.36-31.74 µm x 8.34-14.48 µm (Table 10). The average maximum conidial length (31.74 µm) observed in Am-13 was significantly higher than other isolates, while minimum conidial length (21.36 µm) in Am-16 was at par with Am-15

Table 9: Variability in the conidial septation of *Alternaria mali* isolates

Isolate	Transverse septa (No.)		Longitudinal septa (No.)	
	Range	Mean*	Range	Mean*
Am-1	1-4	2.26	0-3	1.12
Am-2	1-4	2.09	0-3	0.36
Am-3	1-4	2.42	0-4	1.00
Am-4	1-5	2.45	0-3	1.00
Am-5	1-6	2.75	0-4	0.91
Am-6	1-5	2.95	0-3	1.04
Am-7	1-4	2.30	0-3	0.45
Am-8	1-4	1.76	0-3	0.33
Am-9	1-5	2.20	0-4	0.76
Am-10	1-4	2.19	0-3	0.42
Am-11	1-6	2.55	0-3	0.92
Am-12	1-5	2.61	0-4	0.92
Am-13	1-5	2.92	0-3	0.89
Am-14	1-4	2.61	0-3	0.22
Am-15	1-4	2.00	0-3	0.64
Am-16	1-5	2.30	0-3	0.46
Am-17	1-4	2.18	0-3	0.76
Am-18	1-7	2.78	0-3	0.89
Am-19	1-4	2.29	0-3	0.54
Am-20	1-4	2.33	0-3	0.51
Am-21	1-5	2.77	0-3	0.70
$CD_{(P = 0.05)}$		0.24		0.11

* Average of 150 observations

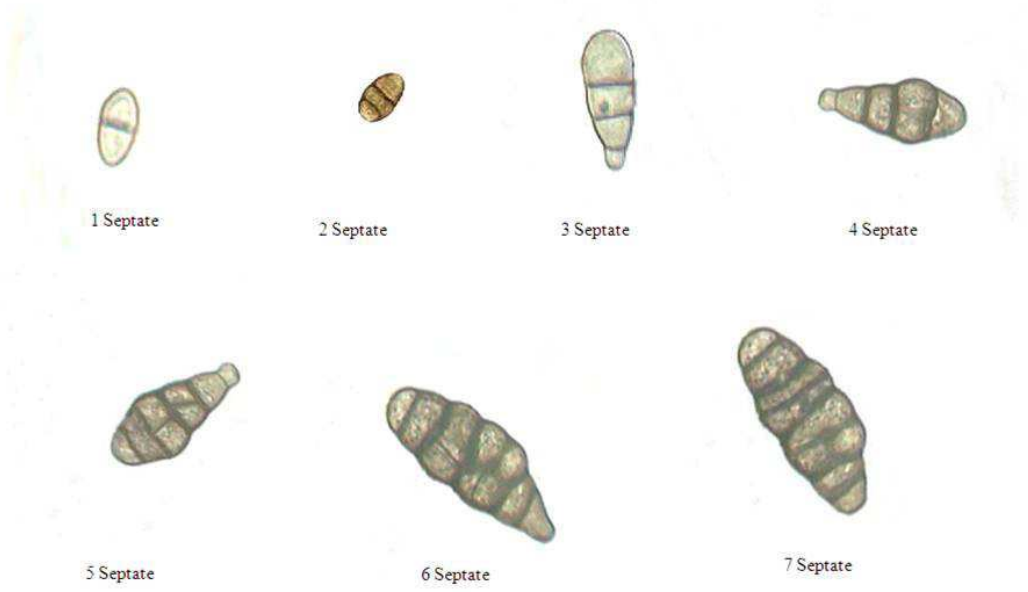


Plate 6a: Transverse spore septation of *Alternaria mali* (1-7)

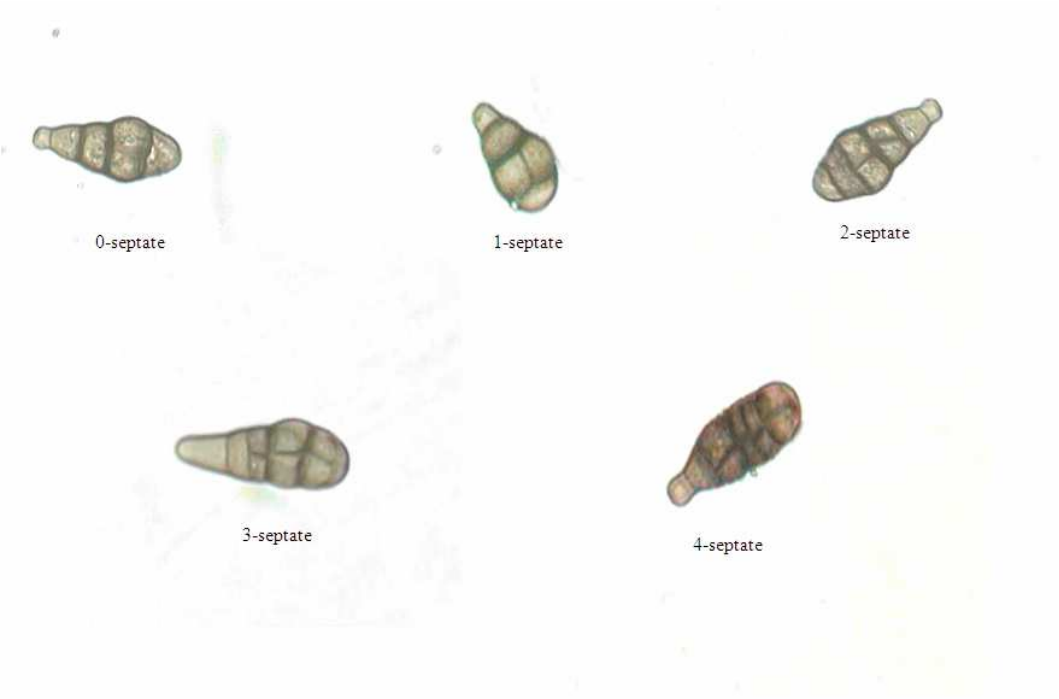


Plate 6b: Longitudinal spore septation of *Alternaria mali* (0-4)

Table 10 : Variability in the conidial size of *Alternaria mali* isolates

Isolate	Length (µm)		Breadth (µm)	
	Range	Mean*	Range	Mean*
Am-1	11.32 - 28.17	26.73	8.23 - 16.01	12.01
Am-2	10.01 - 25.71	23.32	6.22 - 15.82	10.67
Am-3	11.57 - 31.17	27.17	8.12 - 16.07	11.08
Am-4	14.16 - 39.06	26.71	6.63 - 13.40	9.97
Am-5	11.08 - 26.73	23.92	7.66 - 12.24	10.14
Am-6	10.57 - 24.78	22.74	6.87 - 13.42	9.89
Am-7	11.10 - 25.22	22.99	6.23 - 14.42	9.41
Am-8	9.79 - 22.85	22.26	5.57 - 14.28	9.63
Am-9	18.73 - 34.84	26.66	9.03 - 20.02	14.48
Am-10	12.35 - 26.89	23.57	7.68 - 13.53	10.42
Am-11	12.58 - 35.41	26.92	8.04 - 14.49	10.84
Am-12	9.72 - 32.23	26.49	6.04 - 15.35	11.02
Am-13	19.10 - 49.12	31.74	7.68 - 14.75	11.66
Am-14	11.04 - 29.27	24.91	6.93 - 13.87	9.83
Am-15	10.52 - 24.41	21.91	6.21 - 15.05	10.15
Am-16	10.46 - 28.38	21.36	6.58 - 12.26	8.34
Am-17	15.72 - 31.46	28.13	8.57 - 17.39	13.08
Am-18	9.95 - 27.23	22.44	7.73 - 16.01	11.40
Am-19	11.06 - 31.00	25.64	7.78 - 15.21	11.03
Am-20	17.19 - 31.03	26.33	9.19 - 14.67	11.87
Am-21	12.13 - 35.89	28.05	8.12 - 16.25	12.15
$CD_{(P = 0.05)}$		2.52		2.03

* Average of 150 observations

(21.91 µm), Am-8 (22.26 µm), Am-18 (22.44 µm), Am-6(22.74 µm), Am-7 (22.99 µm), Am-2 (23.32 µm) and Am-10 (23.57 µm). Average maximum conidial breadth of 14.48 µm was observed in Am-9 which was at par with 13.08 µm in Am-17 but significantly higher than other isolates, while the minimum conidial breadth of 8.34 µm observed in Am-16 was at par with 9.41 µm in Am-7, 9.63 µm in Am-8, 9.83 µmin Am-14, 9.89 µm in Am-6, 9.97 µm in Am-4, 10.14 µm in Am-5 and 10.15 µm in Am-15. Least conidial length (9.72 µm) and breadth (5.57 µm) was in Am-12 and Am-8, respectively while highest length and breadth was in Am-13 (49.12 µm) and Am-9 (20.02 µm), respectively.

4.2.4.5 Colour morphology

The colour of hyphae, conidiophore and conidia in isolates varied around olive colour (Table 11). Hyphae of seven isolates *viz*., Am-1, Am-2, Am-6, Am-9, Am-10, Am-11 and Am-15 were nearly hyaline, and five isolates (Am-3, Am-4, Am-17, Am-18 and Am-21) were dark olive. Hyphae of four isolates (Am-5, Am-7, Am-12 and Am-16) were olive green and five isolates (Am-8, Am-13, Am-14, Am-19 and Am-20) had light olive green hyphae. Nearly hyaline conidiophores were observed in Am-1, Am-2, Am-6, Am-7, Am-10, Am-16, Am-20 and Am-21 and dark olive in Am-4, Am-5, Am-13, Am-15 and Am-19. Conidiophores were olive green

Table 11 : Variability in colour morphology and conidial shape of *Alternaria mali* isolates

Isolate	Color morphology of *Alternaria mali*			Conidial shape
	Hyphae	Conidiophore	Conidia	
Am-1	NH	NH	LOG	Muriform, obclavate, ovate to round
Am-2	NH	NH	OG	Muriform, obclavate, oval to ellipsoidal
Am-3	DO	LOG	OG	Muriform, obclavate, ovate to round
Am-4	DO	DO	DO	Muriform, obclavate, oval to ellipsoidal
Am-5	OG	DO	DOG	Muriform, obclavate, ovate to round
Am-6	NH	NH	LOG	Muriform, obclavate, oval to ellipsoidal
Am-7	OG	NH	DOG	Muriform, obclavate, ovate to round
Am-8	LOG	LOG	LOG	Muriform, obclavate, pyriform
Am-9	NH	OG	OG	Muriform, obclavate, ovate to round
Am-10	NH	NH	LOG	Muriform, obclavate, ovate to round
Am-11	NH	OG	DOG	Muriform, obclavate, ovate to round
Am-12	OG	LOG	DO	Muriform, obclavate, oval to ellipsoidal
Am-13	LOG	DO	DO	Muriform, obclavate, oval to ellipsoidal
Am-14	LOG	LOG	DOG	Muriform, obclavate, pyriform
Am-15	NH	DO	DOG	Muriform, obclavate, pyriform
Am-16	OG	NH	OG	Muriform, obclavate, pyriform
Am-17	DO	LOG	DO	Muriform, obclavate, ovate to round
Am-18	DO	LOG	OG	Muriform, obclavate, ovate to round
Am-19	LOG	DO	OG	Muriform, obclavate, oval to ellipsoidal
Am-20	LOG	NH	DO	Muriform, obclavate, pyriform
Am-21	DO	NH	DO	Muriform, obclavate, pyriform

Dark olive: DO; Dark olive green: DOG; Nearly hyaline: NH; Olive green: OG; Light olive green: LOG

in Am-9 and Am-11 and light olive green in Am-3, Am-8, Am-12, Am-14, Am-17 and Am-18. Conidia of four isolates (Am-1, Am-6, Am-8 and Am-10) were light olive green, six isolates (Am-2, Am-3, Am-9, Am-16, Am-18 and Am-19) olive green, six isolates (Am-4, Am-12, Am-13, Am-17, Am-20 and Am-21) dark olive and five isolates (Am-5, Am-7, Am-11, Am-14 and Am-15) dark olive green.

4.2.4.6 Conidial shape

All the isolates had muriform and obclavate type of conidia and were ovate to round, oval to ellipsoidal and pyriform in shape (Table 11). Nine isolates viz. Am-1, Am-3, Am-5, Am-7, Am-9, Am-10, Am-11, Am-17 and Am-18 had ovate to round, six isolates (Am-2, Am-4, Am-6, Am-12, Am-13 and Am-19) had oval to ellipsoidal and six (Am-8, Am-14, Am-15, Am-16, Am-20 and Am-21) had pyriform shaped conidia.

4.2.5 Pathogenic variability

A considerable pathogenic variability in incubation period, number of lesions and size of lesions among the isolates was observed (Table 12). The incubation period varied from 2.0 to 6.3 days with the minimum in Am-9, Am-13 and Am-15 which was at par with 2.3 days in Am-4 and Am-8 and 2.6 days in Am-1. The maximum incubation period of 6.3 days was observed in Am-17 which was at par with Am-5 and Am-6.

Table 12: Pathogenic variability in *Alternaria mali* isolates

Isolate	Pathogenic variability		
	Incubation period (days)	No. of lesions*	Size of lesions (mm)* Mean (Range)
Am-1	2.6	12.0	10.2 (7-12)
Am-2	3.6	6.3	9.1 (5-11)
Am-3	4.6	10.0	4.6 (3-6)
Am-4	2.3	10.0	8.5 (4-9)
Am-5	6.0	8.3	6.1 (4-8)
Am-6	5.6	11.0	3.4(2-5)
Am-7	3.0	9.6	5.6 (3-7)
Am-8	2.3	11.6	6.2 (3-8)
Am-9	2.0	11.6	5.8 (3-7)
Am-10	4.0	6.6	6.4 (2-7)
Am-11	3.3	12.3	8.3 (4-10)
Am-12	5.3	9.0	4.5 (2-5)
Am-13	2.0	13.0	5.7 (4-7)
Am-14	3.3	12.0	6.2 (2-7)
Am-15	2.0	12.3	3.1 (2-4)
Am-16	5.0	14.3	4.3 (3-6)
Am-17	6.3	6.0	2.9 (2-4)
Am-18	3.3	8.6	5.6 (4-7)
Am-19	3.0	8.3	5.1 (3-6)
Am-20	4.3	11.3	3.3 (3-5)
Am-21	5.0	12.0	4.6 (2-6)
$CD_{(P = 0.05)}$	0.68	2.41	0.74

*After 10 days of inoculation

The number of lesions varied from 6.3 to 14.3 with the least in Am-2 and highest in Am-16. Am-2 was at par with Am-10 (6.6 lesions), Am-5, Am-19 (8.3 lesions) and Am-18 (8.6 lesions). Similarly Am-16 was at par with Am-13 (13 lesions), Am-11, Am-15 (12.3 lesions) and Am-1, Am-14, Am-21 (12 lesions).

The mean size of lesions produced by isolates varied from 2.9 to 10.2 mm. The minimum lesion size was recorded in Am-17 which was at par with Am-15 (3.1 mm), Am-20 (3.3 mm) and Am-6 (3.4 mm). The maximum lesion size of 10.2 mm observed in Am-1 was significantly different from all the isolates. Overall, the lesion size varied from 2-12 mm with maximum in Am-1 and least in Am-6, Am-10, Am-12, Am-14, Am-15, Am-17 and Am-21. Am-1 was highly virulent as compared to other isolates with the incubation period of 2.6 days, number of lesions 12 and maximum size of lesions (10.2 mm).

4.2.6 Similarity of isolates on the basis of cultural, morphological and pathogenic characters

The similarity percentage of isolates ranged from 57.6 to 82.1 with the minimum between Am-10 and Am-21, and maximum between Am-2 and Am-10 and between Am-4 and Am-11 (Table 13). The dendrogram analysis of 21 isolates revealed diversity within *A. mali* population (Fig. 2). At 66 per cent similarity coefficient, all the isolates formed one single

Table 13. Percentages of cultural, morphological and pathogenic similarities between different *Alternaria mali* isolates

	Am-1	Am-2	Am-3	Am-4	Am-5	Am-6	Am-7	Am-8	Am-9	Am-10	Am-11	Am-12	Am-13	Am-14	Am-15	Am-16	Am-17	Am-18	Am-19	Am-20	Am-21
Am-1	-																				
Am-2	67.5	-																			
Am-3	74.1	62.9	-																		
Am-4	75.4	68.2	73.5	-																	
Am-5	64.2	72.8	67.5	72.8	-																
Am-6	63.5	66.8	65.5	64.2	67.5	-															
Am-7	66.2	69.5	66.8	64.2	68.8	73.5	-														
Am-8	69.5	66.2	67.5	70.1	65.5	72.8	67.5	-													
Am-9	69.5	68.8	64.9	64.9	64.2	67.5	67.5	69.5	-												
Am-10	64.2	82.1	63.5	58.2	70.8	67.5	75.4	64.2	76.1	-											
Am-11	73.5	75.4	72.8	82.1	70.8	67.5	62.2	68.2	72.1	65.5	-										
Am-12	66.8	67.5	71.5	72.8	68.2	62.2	58.2	68.2	61.5	61.5	77.4	-									
Am-13	65.5	64.9	64.9	67.5	69.5	67.5	64.9	74.8	69.5	65.5	70.8	76.1	-								
Am-14	65.5	70.1	62.2	70.1	70.8	66.2	64.9	70.8	70.8	64.2	74.8	74.8	70.8	-							
Am-15	64.9	76.1	58.9	69.5	67.5	70.8	61.5	70.1	68.8	67.5	74.1	72.8	71.5	70.1	-						
Am-16	68.2	71.5	62.2	66.2	65.5	63.5	71.5	65.5	69.5	70.8	66.8	68.2	64.2	65.5	72.8	-					
Am-17	74.1	64.2	65.5	65.5	59.6	64.2	65.5	67.5	71.5	60.9	66.2	64.9	63.5	64.9	73.5	74.1	-				
Am-18	62.9	76.8	70.1	68.8	73.5	71.5	71.5	65.5	64.2	69.5	70.8	66.8	66.8	69.5	68.8	68.2	67.5	-			
Am-19	66.8	68.8	74.1	67.5	69.5	63.5	68.8	60.2	64.2	65.5	66.8	68.2	62.9	62.9	63.5	62.9	66.2	70.8	-		
Am-20	73.5	60.9	72.8	66.2	62.9	64.9	68.8	60.2	70.8	68.2	68.2	61.5	65.5	68.2	62.2	66.8	70.1	64.2	76.1	-	
Am-21	72.1	59.6	72.8	66.2	62.9	66.2	62.2	64.2	66.8	57.6	70.8	70.8	66.8	66.8	64.9	65.5	76.8	66.8	72.1	76.1	-

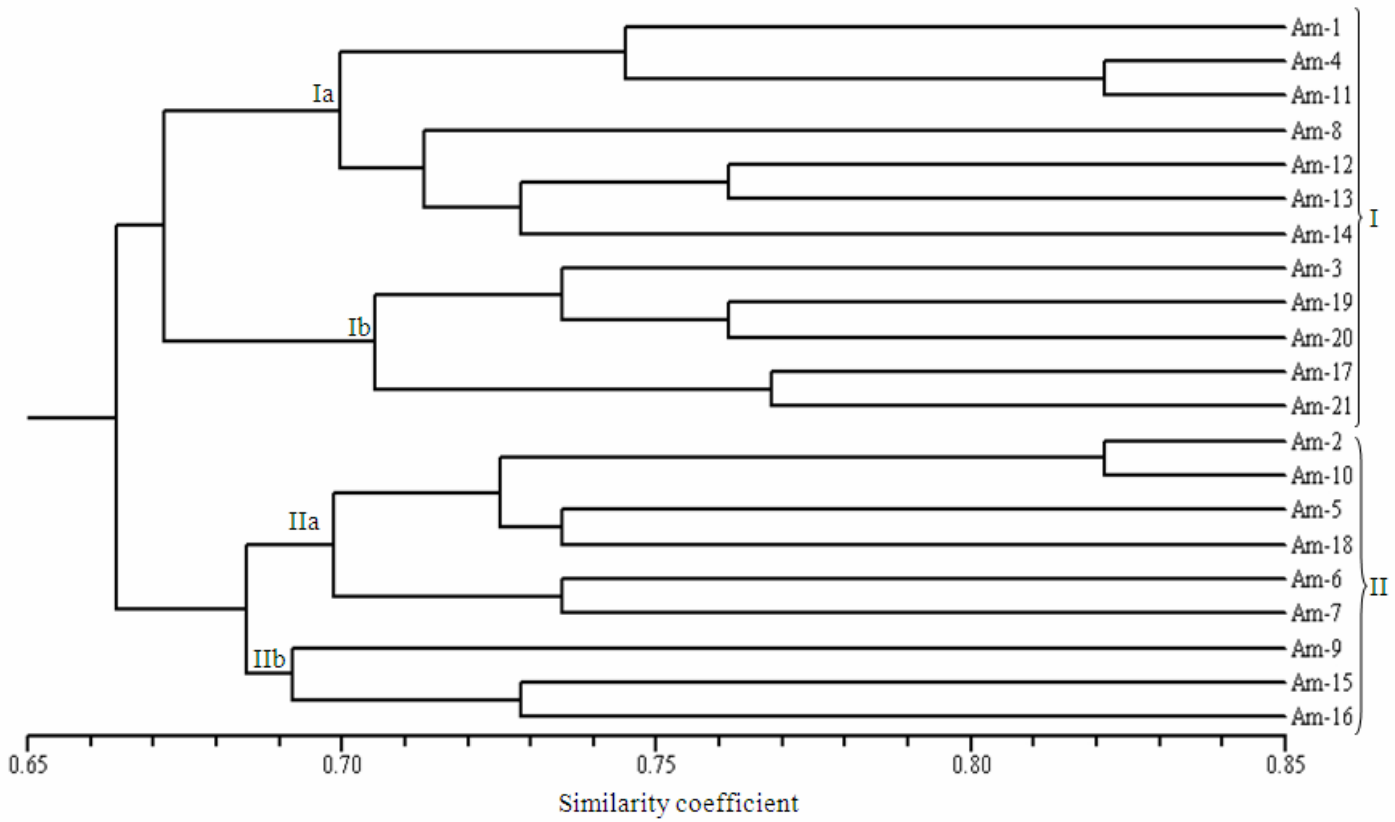


Fig. 2: Dendrogram of 21 isolates of *Alternaria mali* generated by UPGMA (Unweighted pair group method arithmetic mean) analysis of cultural, morphological and pathogenic characters. Scale at the bottom depicts the similarity values obtained using similarity coefficient

group. At 67 per cent similarity coefficient, all the isolates were categorized into 2 clusters (I and II). Cluster I contained 12 isolates, while as cluster II accommodated 9 isolates. Cluster I was further subdivided into Ia and Ib accommodating 7 and 5 isolates, respectively. Similarly, cluster II was also subdivided into IIa and IIb accommodating 6 and 3 isolates, respectively. The tendency to group isolates in relation to their location was not obtained, as the two major clusters (I and II) and their sub-clusters (Ia, Ib and IIa, IIb) contained isolates from diverse regions.

4.2.7 Molecular variability

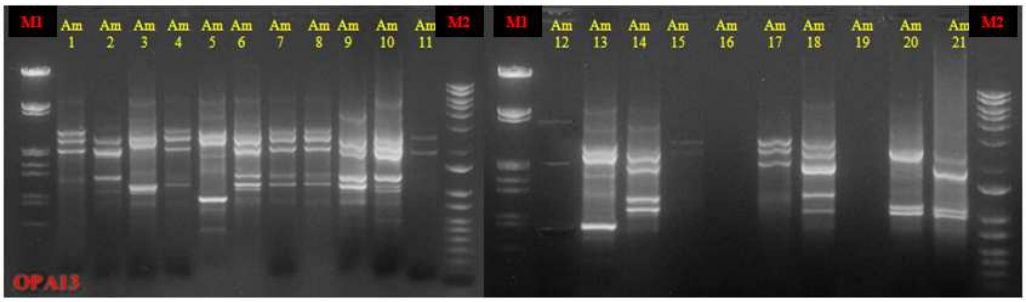
Thirty RAPD 10-mer and twelve 20-mer primers (Table 14) were screened by amplifying two randomly selected fungal isolates with a view to select those which yield maximum consistency in polymorphism. Among the 42 primers, one 10-mer [OPA-13 (5' CAG CAC CCA C 3')] and three 20-mers [U2 (5' CCC AGC AAC TGA TCG CAC AC 3'), U3 (5' GTG TGC GAT CAG TTG CTG GG 3') and U9 (5' AAT GTG TGG CAA GCT GGT GG 3')] produced consistent polymorphic bands and were selected for fingerprinting of 21 isolates of *A. mali* (Plate 7). The number of scorable and polymorphic bands ranged from 7 to 14 and 6 to 14, respectively (Table 15). Highest scored bands were 14 in U3 showing 100 per cent polymorphism. The least scorable bands were 7 in U2 with 6 polymorphic

Table 14 : Nucleotide sequences of primers used in RAPD

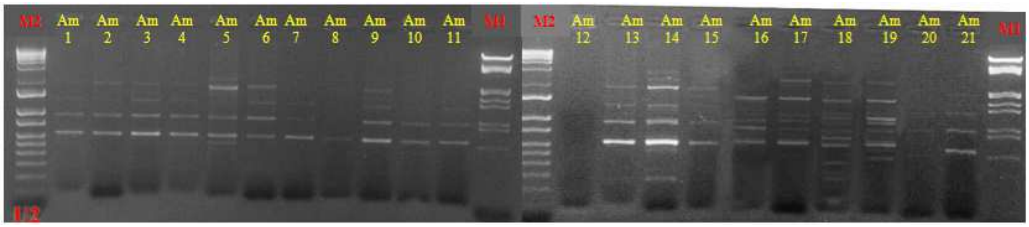
Primer name	Sequence (5' to 3')	Primer name	Sequence (5' to 3')
OPA-01	CAG GCC CTT C	OPC-06	GAA CGG ACT C
OPA-02	TGC CGA GCT G	OPC-07	GTC CCG ACG A
OPA-03	ATG CAG CCA C	OPC-08	TGG ACC GGT G
OPA-04	AAT CGG GCT G	OPC-09	CTC ACC GTC C
OPA-05	AGG GGT CTT G	OPC-11	AAA GCT GCG G
OPA-06	GGT CCC TGA C	OPC-12	TGT CAT CCC C
OPA-07	GAA ACG GGT G	OPC-13	AAG CCT CGT C
OPA-08	GTG ACG TAG G	OPC-14	TGC GTG CTT G
OPA-09	GGG TAA CGC C	OPC-15	GAC GGA TCA G
OPA-10	GTG ATC GCG T	U1	ATC CAA GGT CCG AGA CAA CC
OPA-11	CAA TCG CCG T	U2	CCC AGC AAC TGA TCG CAC AC
OPA-12	TCG GCG ATA G	U3	GTG TGC GAT CAG TTG CTG GG
OPA-13	CAG CAC CCA C	U4	AGG ACT CGA TAA CAG GCT CC
OPA-14	TCT GTG CTG G	U5	GGC AAG CTG GTG GGA GGT AC
OPA-15	TTC CGA ACC C	U6	ATG TGT GCG ATC AGT TGC TG
OPA-16	AGC CAG CGA A	U7	GGT GAA CAG TGA GAT GAA CC
OPA-17	GAC CGC TTG T	U8	TAC ATC GCA AGT GAC ACA GG
OPC-01	TTC GAG CCA G	U9	AAT GTG TGG CAA GCT GGT GG
OPC-03	GGG GGT CTT T	U10	GAT GTG TTC TTG GAG CCT GT
OPC-04	CCG CAT CTA C	U11	GGA CAA GAA GAG GAT GTG GA
OPC-05	GAT GAC CGC C	U12	GGT TGT AGG CCG ATA TTG TC

Table 15: Number of scorable and polymorphic RAPD bands generated by PCR amplification

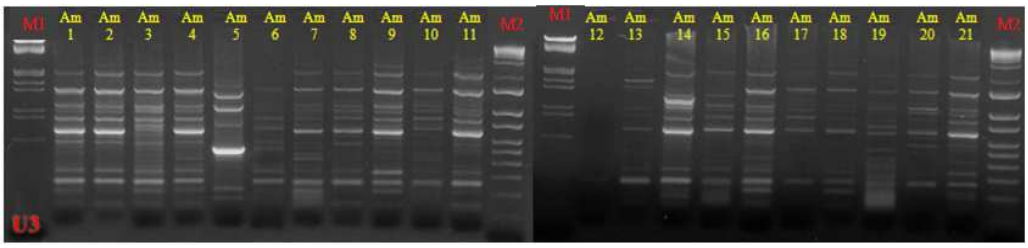
Primer	Scored bands	Polymorphic bands	Polymorphism (%)
OPA-13	10	10	100
U2	7	6	85.71
U3	14	14	100
U9	11	11	100
Total	**42**	**41**	**97.61**



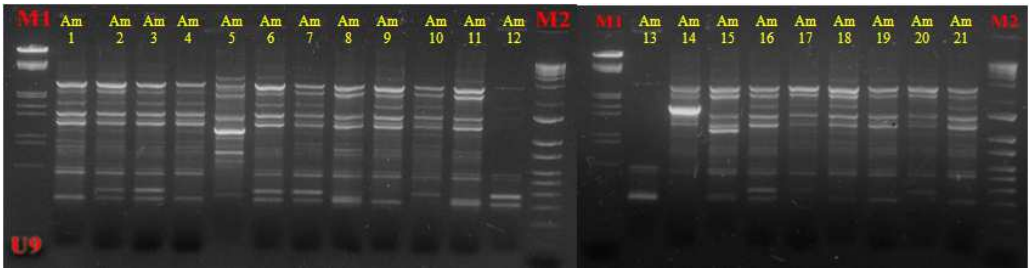
I. RAPD profile of 21 *A. mali* isolates with OPA-13 primer



II. RAPD profile of 21 *A. mali* isolates with U-2 primer



III. RAPD profile of 21 *A. mali* isolates with U-3 primer



IV. RAPD profile of 21 *A. mali* isolates with U-9 primer

Plate 7: **Gel eletrophoresis of the RAPD amplified products from *A. mali* obtained with different primers. Column M1 and M2 shows the band pattern for the fragment size markers (100 bp and Lambda DNA/*EcoR1+Hind III*)**

bands. All the primers showed 100 per cent polymorphism, except U2 with 85.71 per cent polymorphism. In total 42 bands were scored of which 41 were polymorphic thereby showing 97.61 per cent polymorphism.

Cluster analysis of RAPD bands of all the primers generated a dendrogram, which showed genetic diversity within *A. mali* isolates (Fig. 3). At 57.5 per cent Dice similarity coefficient all the isolates form a single clade, thus showing 42.5 per cent dissimilarity among the isolates. Using 64 per cent Dice similarity coefficient as a cutoff point, all the isolates formed two RAPD fingerprint groups. Cluster A contained 19 isolates, while cluster B accommodated two isolates only *viz*., Am-5 and Am-19. At 68 per cent Dice similarity coefficient, all the isolates were categorized into 5 clusters (I to V). Cluster I was further subdivided into Ia and Ib groups accommodating 9 (Am-1, Am-21, Am-4, Am-2, Am-9, Am-3, Am-10, Am-8 and Am-16) and 2 (Am-11 and Am-15) isolates, respectively. The tendency to group isolates in relation to their location was not obtained, as the two major clusters (A and B) as well as the 5 clusters (I to V) and sub-clusters (Ia and Ib) contained isolates from diverse regions. Similarity percentage of the isolates ranged from 38.0 to 91.2 with minimum similarity between Am-5 and Am-7 and maximum between Am-1 and Am-21 (Table 16). The maximum similarity of 91.2 per cent was followed by

Table 16 : Percentages of genetic similarities between different *Alternaria mali* isolates

	Am-1	Am-2	Am-3	Am-4	Am-5	Am-6	Am-7	Am-8	Am-9	Am-10	Am-11	Am-12	Am-13	Am-14	Am-15	Am-16	Am-17	Am-18	Am-19	Am-20	Am-21
Am-1	-																				
Am-2	82.7	-																			
Am-3	77.1	77.1	-																		
Am-4	90.9	87.2	74.0	-																	
Am-5	50.9	43.1	60.0	41.6	-																
Am-6	73.0	76.9	74.5	73.4	44.4	-															
Am-7	65.3	77.5	62.5	73.9	38.0	69.7	-														
Am-8	83.6	76.3	66.6	80.7	50.0	61.2	69.5	-													
Am-9	82.1	85.7	83.6	86.7	53.0	76.0	72.3	71.6	-												
Am-10	80.0	83.6	74.0	76.9	50.0	69.3	69.5	73.0	75.4	-											
Am-11	76.0	76.0	69.3	80.8	41.8	63.6	68.2	72.3	75.0	59.5	-										
Am-12	67.7	71.1	75.8	60.7	61.5	52.8	48.0	64.2	66.6	60.7	58.8	-									
Am-13	74.0	70.3	64.1	70.5	55.3	79.1	66.6	66.6	69.2	70.5	60.8	54.5	-								
Am-14	66.6	70.3	67.9	66.6	55.3	54.1	57.7	66.6	69.2	58.8	69.5	69.0	56.0	-							
Am-15	73.9	69.5	66.6	74.4	41.0	50.0	54.0	69.7	72.7	60.4	78.9	59.5	47.6	66.6	-						
Am-16	76.6	76.6	74.5	77.1	49.0	62.9	70.5	77.1	75.8	73.6	65.3	68.8	67.8	67.8	54.1	-					
Am-17	69.3	65.3	62.5	65.2	47.6	60.4	50.0	65.2	68.0	60.8	58.5	68.0	57.7	75.5	70.2	54.9	-				
Am-18	66.6	62.7	64.0	62.5	50.0	71.1	61.9	58.3	69.3	62.5	55.8	61.5	68.0	63.8	51.2	64.1	76.1	-			
Am-19	71.1	67.7	62.0	64.2	69.2	64.1	64.0	71.4	66.6	67.8	50.9	66.6	72.7	69.0	46.8	72.1	68.0	65.3	-		
Am-20	69.0	80.0	74.0	69.2	50.0	65.3	60.8	65.3	75.4	73.0	68.0	75.0	62.7	82.3	65.1	63.1	78.2	66.6	67.8	-	
Am-21	91.2	84.2	85.7	88.8	52.0	70.5	66.6	77.7	87.2	77.7	77.5	68.9	67.9	67.9	75.5	74.5	66.6	60.0	68.9	74.0	-

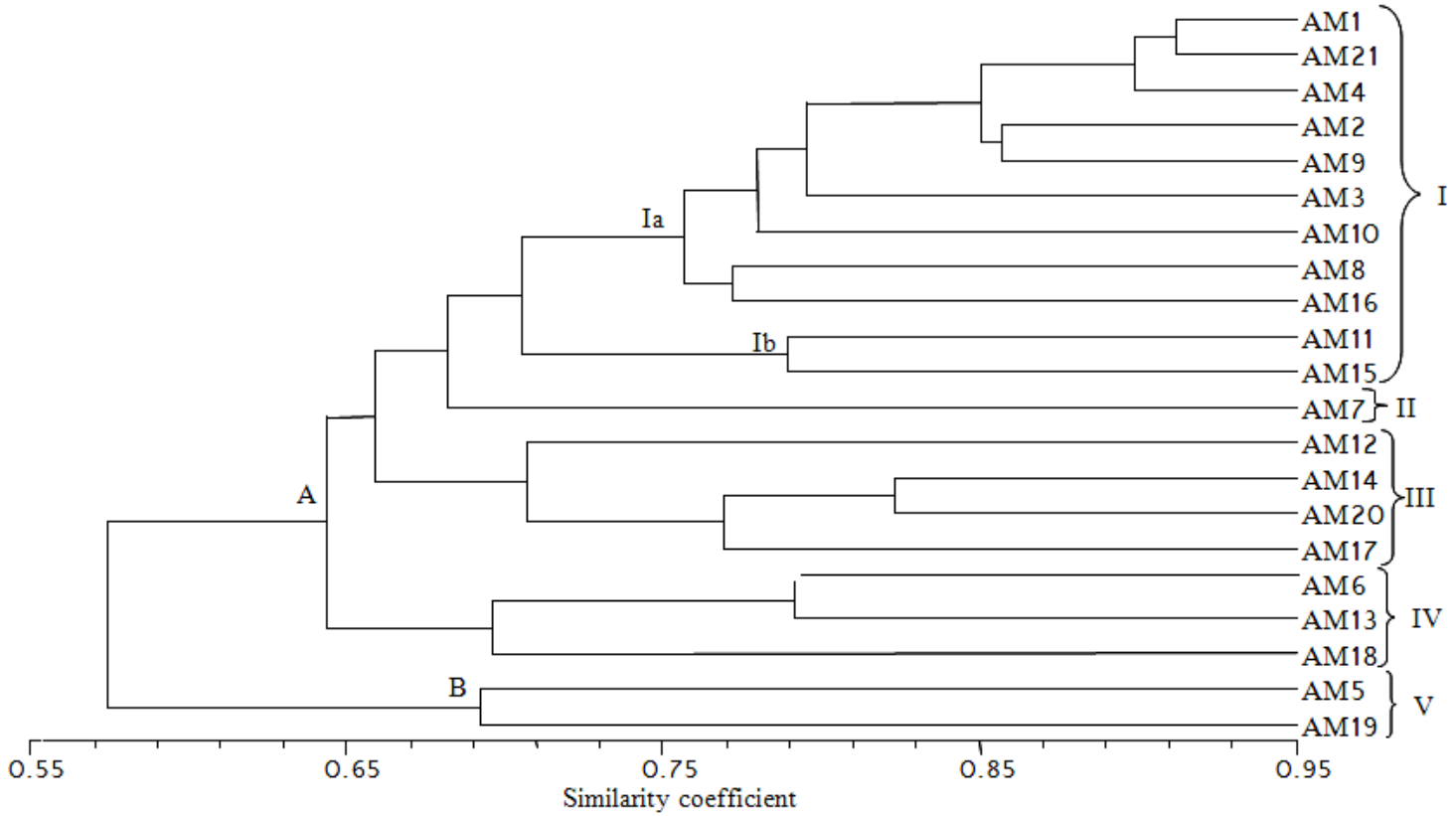


Fig. 3: Dendrogram of 21 isolates of *Alternaria mali* generated by UPGMA (Unweighted pair group method arithmetic mean) analysis of RAPD bands obtained with four random primers. Scale at the bottom depicts the similarity values obtained using Dice similarity coefficient

90.9 per cent between Am-1 and Am-4. All the isolates were 50 per cent or more genetically similar to each other except Am-4 and Am-5 (41.6%), Am-5 and Am-6 (44.4%), Am-5 and Am-7 (38.0%), Am-5and Am-11 (41.8%), Am-5 and Am-15 (41.0%), Am-5 and Am-16 (49.0%), Am-5 and Am-17 (47.6%), Am-7 and Am-12 (48.0%), Am-13 and Am-15 (47.6%) and Am-15 and Am-19 (46.8%).

4.3 Screening of cultivars

Screening of 13 apple cultivars against *A. mali* revealed that all the cultivars succumbed to the disease with varied magnitude of intensity (Table 17). In 2008 mean disease intensity (13.55%) was significantly higher than recorded in 2009 (10.88%). Amongst cultivars, Red Delicious had significantly highest disease intensity of 30.49 per cent in 2008 and 26.24 per cent in 2009, while White Dotted Red showed the least disease intensity of 0.88 and 0.45 per cent during the same period. However, the disease intensity of both the cultivars was significantly different from all other cultivars. Two years pooled data revealed the highest disease intensity of 28.36 per cent in Red Delicious followed by 22.04 per cent in Red Gold, 16.75 per cent in Gulshan and 14.81 per cent in Lal Ambri. All the above cultivars differed significantly from each other. This was followed by 13.47 per cent in Golden Delicious and 13.29 per cent in Sunheri both statistically

Table 17. Evaluation of apple cultivars for their response to *Alternaria mali* infection

Cultivar	Disease intensity (%)		
	2008	**2009**	**Pooled**
Akbar	12.77 (20.92)	10.45 (18.84)	**11.61 (19.88)**
American Apirouge	4.68 (12.48)	3.17 (10.22)	**3.92 (11.35)**
Firdous	7.41 (15.79)	6.34 (14.55)	**6.87 (15.17)**
Golden Delicious	14.29 (22.21)	12.65 (20.81)	**13.47 (21.51)**
Gulshan	19.41 (26.14)	14.09 (22.03)	**16.75 (24.08)**
Lal Ambri	16.38 (23.86)	13.25 (21.32)	**14.81 (22.59)**
Red Delicious	30.49 (33.51)	26.24 (30.80)	**28.36 (32.15)**
Red Gold	23.33 (28.88)	20.76 (27.09)	**22.04 (27.99)**
Shireen	8.28 (16.71)	7.19 (15.53)	**7.73 (16.12)**
Spartan	9.33 (17.74)	5.38 (13.37)	**7.35 (15.55)**
Star Crimson	13.51 (21.56)	10.42 (18.81)	**11.96 (20.18)**
Sunheri	15.47 (23.16)	11.12 (19.46)	**13.29 (21.31)**
White Dotted Red	0.88 (5.34)	0.45 (3.66)	**0.66 (4.50)**
Mean	**13.55 (20.64)**	**10.88 (18.19)**	-
$CD_{(P = 0.05)}$ Cultivar : 0.82; Year : 0.32; Cultivar x Year : 1.17			

Values in parenthesis are arcsine transformed

at par. Cultivar Star Crimson and Akbar with disease intensity of 11.96 and 11.61 per cent were at par with each other but significantly different from other cultivars. Disease intensities of 7.73 and 7.35 per cent recorded in Shireen and Spartan were at par with each other and significantly less than recorded in Star Crimson and Akbar however, Spartan was at par with Firdous (6.87%). This was followed by 3.92 per cent in American Apirouge and 0.66 per cent in White Dotted Red which was least effected cultivar.

Evaluated cultivars were arbitrarily categorized into five groups on the basis of disease intensities recorded during 2008 and 2009 (Table 18). White Dotted Red was highly resistant with the PDI (percent disease index) between 0.1-1.0. American Apirouge with the PDI 1.1-5.0 was rated as resistant, while the three cultivars (Firdous, Shireen and Sparten) with the PDI in the range of 5.1-10.0 were rated as moderately resistant. Six cultivars (Akbar, Golden Delicious, Gulshan, Lal Ambri, Star Crimson and Sunheri) with the PDI between 10.1 and 20.0 were rated as susceptible and the two cultivars Red Delicious and Red Gold with the PDI more than 20.0 were rated as highly susceptible.

Table 18 : Grouping of various apple cultivars into different reaction categories on the basis of their response to Alternaria leaf blotch

Reaction category	Percent disease index (PDI)	Cultivar
Highly resistant	0.1 - 1.0	White Dotted Red
Resistant	1.1 - 5.0	American Apirouge
Moderately resistant	5.1 - 10.0	Firdous, Shireen, Spartan
Susceptible	10.1 - 20.0	Akbar, Golden Delicious, Gulshan, Lal Ambri, Star Crimson, Sunheri
Highly susceptible	> 20.0	Red Delicious, Red Gold

4.4 Efficacy of SAR activators for the management of disease

Evaluation of SAR activators along with fungicide Penconazole as standard check at three different concentrations before and after pathogen inoculation indicated that all the treatments significantly lowered disease intensity as compared to check (water spray) (Table 19). The disease intensity ranged from 5.88 to 17.34 per cent in chemical (SAR activators) treatments in comparison to 35.35 per cent in check indicating that all the SAR chemicals were significantly effective in lowering the disease intensity. The least disease intensity of 5.88 per cent was recorded in BABA (β-aminobutyric acid) which was at par with penconazole (standard) having a disease intensity of 5.56 per cent. However both were significantly lower than other chemicals. These were followed by K_3PO_4, BTH (benzothiadiazole S-methyl ester), INA (2,6-dichloroisonicotinic acid), $Ca(OH)_2$, K_2HPO_4 and $CaCO_3$ with disease intensity of 7.12, 11.02, 12.38, 14.07, 15.14 and 17.34 per cent, respectively.

The application of SAR activators before pathogen inoculation showed significantly lower disease intensity (12.69%) in comparison to SAR application after pathogen inoculation (14.83%). However INA and penconazole (standard check) were statistically at par in both cases of SAR application. All the three concentrations of each chemical significantly

Table 19 : Efficacy of various SAR activators against Alternaria leaf blotch of apple cv.Red Delicious

Treatment	Conc. (ppm)/ *: mM	Disease intensity		
		Before inoculation	After inoculation	Mean
INA (2,6-dichloroisonicotinic acid)	50	14.42	16.56	**15.49**
	100	11.50	12.30	**11.90**
	200	9.28	10.22	**9.75**
	Mean	**11.73**	**13.02**	12.38
BTH (benzothiadiazole S-methyl ester)	50	11.40	14.75	**13.07**
	100	9.77	12.83	**11.30**
	200	7.99	9.42	**8.70**
	Mean	**9.72**	**12.33**	11.02
BABA (β-aminobutyric acid)	500	9.00	9.93	**9.46**
	1000	4.65	6.23	**5.44**
	2000	1.47	4.03	**2.75**
	Mean	**5.04**	**6.73**	5.88
*K_2HPO_4	25	16.39	19.37	**17.88**
	50	15.04	16.24	**15.64**
	100	10.82	12.97	**11.90**
	Mean	**14.08**	**16.19**	15.14
*K_3PO_4	25	6.90	10.86	**8.88**
	50	6.21	8.89	**7.55**
	100	4.39	5.48	**4.93**
	Mean	**5.83**	**8.41**	7.12
*$Ca(OH)_2$	25	15.34	21.27	**18.31**
	50	10.72	16.51	**13.61**
	100	10.03	10.54	**10.29**
	Mean	**12.03**	**16.11**	14.07
*$CaCO_3$	25	18.46	24.13	**21.29**
	50	14.68	21.33	**18.00**
	100	11.65	13.82	**12.73**
	Mean	**14.93**	**19.76**	17.34
Penconazole (Standard)	300	8.49	7.90	**8.19**
	400	5.25	4.79	**5.02**
	500	3.29	3.68	**3.48**
	Mean	**5.67**	**5.45**	5.56
Water Check)	-	35.62	35.98	**35.80**
	-	34.70	35.02	**34.86**
	-	35.41	35.40	**35.40**
	-	**35.24**	**35.47**	35.35
Mean		**12.69**	**14.83**	

$CD_{(P = 0.05)}$			
Chemical	0.61	B/A	0.28
Chemical x Concentration	1.06	Chemical x B/A	0.86
Chemical x Concentration x B/A	1.50		

Values were arcsine transformed before analysis; B/A: Before and after inoculation

differed from each other with the least disease intensity observed in highest concentration of each chemical. All the three concentrations before inoculation recorded significantly lower disease intensity as compared to respective concentrations after inoculation.

Amongst the various treatments, BABA at 2000 ppm was highly effective with least disease intensity of 1.47 per cent when applied before pathogen inoculation. The next best treatments were penconazole applied at the rate of 500 ppm before and after inoculation and BABA at 2000 ppm after inoculation with disease intensity of 3.29, 3.68 and 4.03 per cent, respectively. These were followed by 100 mM K_3PO_4 applied before inoculation, 1000 ppm BABA before inoculation and 400 ppm penconazole after inoculation with disease intensity of 4.39, 4.65 and 4.79 per cent, respectively. The above treatments were statistically at par with each other and significantly inferior to BABA at 2000 ppm before inoculation, but superior to other treatments.

CHAPTER - 5

DISCUSSION

CHAPTER - 5

DISCUSSION

Apple (*Malus domestica* Borkh.) is a predominant fruit crop of Jammu and Kashmir which has attained the status of an industry in the state. Like other horticultural crops, apple is attacked by several pathogens of fungal, bacterial and viral etiology which impair the quality and quantity of the fruit. However, huge crop losses are incurred mostly by fungal diseases. Among these, Alternaria leaf blotch has assumed an alarming threat because of the prevalence of the disease in all the major apple growing areas of Kashmir valley. Shahzad (2003) recorded Alternaria leaf blotch with varied magnitude of disease incidence and intensity in four districts of Kashmir *viz.* Anantnag, Baramulla, Budgam and Pulwama and also observed variation in degree of susceptibility among different apple cultivars. This variation may be attributed to many factors including the pathogen variability. Control of *Alternaria mali* can be achieved through use the of fungicides but some isolates have reportedly developed tolerance to some fungicides such as iprodione, mancozeb and captan (Lee and Kim, 1986; Osanai *et al.*, 1987; Asari and Takahashi, 1988). Presently disease is managed by 6-8 foliar fungicidal sprays posing serious environmental concerns and selection pressure on pathogen population. The use of

resistant cultivars is an alternative to minimize the disease. However, in apple it is difficult to develop resistant cultivars because of long juvenile phase and self incompatibility. Hence, use of systemic acquired resistance (SAR) activators in the disease management seems environmentally safe as compared to current pesticides (Vallad and Goodman, 2004). Unlike traditional pesticides, SAR activators do not exhibit any direct antimicrobial activity but prime the plants for resistance thus providing a safe way to control disease without asserting direct selection pressure on pathogen populations.

Survey conducted during the year 2007 and 2008 in seven districts of Kashmir valley confirms the prevalence of disease in all the apple growing areas of the valley with an overall mean disease incidence and intensity of 42.11 and 21.68 per cent, respectively. The disease was severe during 2008 with overall mean disease incidence and intensity of 45.18 and 24.38 per cent in comparison to 39.05 and 19.00 per cent, respectively, in 2007. This higher disease severity could be attributed to higher inoculum build up because of more favourable climatic conditions in 2008 (RH, 73.2%; RF, 81.5 mm) than in 2007(RH 68.1%, RF 67.5 mm), especially during May to August, the period conducive for the disease development (Appendix-1).

Such conducive environment favouring the disease development has been reported by many researchers (Yoon *et al*., 1989a; Shahzad, 2003).

The disease incidence and intensity in surveyed areas varied from 25.49 to 51.94 and 12.52 to 26.34, respectively, with highest disease incidence and intensity recorded in Pulwama, and lowest in Shopian. The site selection varied from site selection earlier by Shahzad in 2003. In addition three more districts were covered under survey programme. Shahzad (2003) reported that the Alternaria leaf blotch incidence and intensity among the four districts of the valley varied from 31.37 to 43.50 and 12.34 to 22.57, respectively, with lowest in Budgam and highest in Anantnag. Of the sites surveyed, the highest disease incidence of 68.74 per cent was recorded in Batwina (Ganderbal) followed by Pinglena (Pulwama), Sehpora (Kulgam) and Babber (Pulwama), with disease incidence of 55.48, 52.88 and 51.56 per cent, respectively. The highest disease intensity of 33.62 per cent was also recorded in Batwina (Ganderbal) followed by 27.65 per cent in Pinglena (Pulwama), 26.90 per cent in Tarzoo (Baramulla) and 26.81 per cent in Babber (Pulwama). Higher disease incidence and intensity in various districts and sites surveyed could be attributed to higher plant density, besides non-disposal of the fallen diseased leaves and heavy infestation of European red mite

(*Panonychus ulmi*) which facilitates the penetration of *A. mali*. Filajdic *et al*. (1995) observed more disease severity with increased mite population. Occasional or rather neglected spray programme followed in these areas seem to have contributed in building mite population density threshold. The least disease incidence (19.33%) and intensity (9.21%) was recorded in the Kachdoora of district Shopian. The less disease incidence and intensity could be attributed to lesser plant density and better orchard management. Overall, variation in disease severity may be because of the variation in various factors like, altitude, climate, plant age and management practices. The variation in incidence and intensity of Alternaria leaf blotch disease in various locations have previously been reported by Yoon *et al*. (1989a), Filajdic and Sutton (1991), Bulajic *et al*. (1996) and Shahzad (2003). Filajdic and Sutton (1991) reported 0 to 95 per cent Alternaria leaf blotch incidence in 60 apple orchards in western North Carolina.

The causal pathogen, *Alternaria mali* (Roberts), was isolated from diseased leaves of apple cv. Red Delicious and their pathogenicity established on detached leaves. *Alternaria mali* being the weak pathogen is unable to force its entry and hence require wounds to facilitate its penetration into the leaf. Penetration of host plants through wounds has been shown to be a significant component of the infection process for a

number of *Alternaria* species (Rotem, 1994). Thus the method was followed to collect twenty one isolates from different sites. Ozgonen and Karaca (2006) also confirmed pathogenicity of *A. mali* isolates by inoculation with a conidial suspension on detached wounded apple leaves. Similar technique was adopted by Soleimani and Esmailzadeh (2007) while confirming pathogenicity of the isolates of *A. mali* on detached wounded apple leaves in Iran. Slavov *et al*. (2004) reported the wounds or stomata as entry points of less virulent species of *Alternaria*.

Successful execution of a disease management programme, in addition to other factors, depends on the understanding of pathogen population structure and mechanism by which variation arises within populations. Variations in pathogen population can be detected with cultural, morphological, pathogenic and molecular tools. During present work efforts were made to ascertain the prevalence of variation in isolates of *A. mali* from different locations of the valley. Twenty one isolates collected from 21 locations of the valley varied in their cultural, morphological, pathogenic and molecular characteristics. Several workers have also reported cultural, morphological, pathogenic and molecular variability among isolates of *Alternaria* spp. (Saharan and Kadian, 1983;

Khan *et al*, 1992; Vishwanath and Kolte, 1997; Pandey and Vishwakarma, 1999; Singh *et al*, 2003; Kumar *et al*., 2008 and Tetarwal *et al.*, 2008).

Isolates of *Alternaria mali* varied in their cultural characteristics *viz*. colony type, colour, margin, pigmentation and growth rate. Colonies were velvety or cottony and mostly appressed with colour ranging from light to dark olivacious. Present results are in agreement with the observations of Pusz (2009) who found that colonies of *Alternaria alternata* from *Amaranthus retroflexus* varied from light grey to dark grey. Similarly, Rai and Kumari (2009) observed loose, cottony, compact and dense type of colonies with light to dark black colour in *Alternaria alternata* infecting Periwinkle. Quayyum *et al*. (2005) reported among the six isolates of *Alternaria panax* on American ginseng, four isolates produced dense, velvety, dark brown colonies, while colonies of two isolates were appressed, shiny and dark grey to dark greenish in colour. Margins of the colonies were regular to irregular. Colour of margins also varied among the isolates. Ramegowda and Naik (2008) reported colour of isolates of *Alternaria macrospora* colony margin varied from creamy white to brown with irregular to smooth texture. Pryor and Michailides (2002) reported variability in the colony colour, type and margins among *Alternaria* isolates

associated with late blight of pistachio. In the present study colonies were slow growing with varied growth rate (growth/day). Isolate Am-13 grew fast among all the isolates with mean growth rate of 8.21 mm while isolate Am-5 grew slowly with mean growth rate of 5.86 mm. Mortensen and Bergman (1983) reported colony diameter of 35 isolates of *Alternaria carthami* in the range of 32 to 81 mm in 8 days old culture. Isolates in the present study depicted periodic changes in their growth rates. All the isolates showed an increasing trend of growth rate from 2 days to 8 days but decreased afterwards. Thrall *et al*. (2005) also observed considerable variation in the growth rate of *Alternaria brassicicola* isolates. Almost similar observations were recorded by Pusz (2009) on *Alternaria alternata* with colony diameter ranging from 4.8 to 6.8 cm among 26 isolates after 7 days of inoculation. Among the 10 isolates of *Alternaria alternata* colony diameter after 7 days of incubation varied from 16.85 to 32.35 mm (Rai and Kumari, 2009). Isolates were found to impregnate the media with a colour pigment mostly grey to brown with some variation and were clearly visible from under side of the plates. Our observations are comparable to the findings of Hubballi *et al*. (2011) who reported variation in the pigmentation of 15 *Alternaria alternata* isolates producing black, brownish black, greenish black, brown and yellow pigmentation.

Isolates were studied for morphological variation in their hyphae, conidiophore and conidia. Isolates varied in their hyphal dimensions. Mean hyphal width ranged from 3.28 to 4.28 µm with maximum in Am-4 and minimum in Am-21. In conformity to the present results Ramegowda and Naik (2008) observed the hyphal width of 9 isolates of *Alternaria macrospora* varying from 2.87 to 6.95 µm. Variation in hyphal width has also been reported in other fungi (Sunder *et al.*, 2003). In the present study, great variation in conidiophore size was observed with maximum length of 68.18 µm in Am-9 while minimum length of 26.99 µm was observed in Am-17 and mean length varied from 33.01 to 62.58 µm. Similarly, conidiophore breadth varied from 3.30-4.70 µm among the isolates with least breadth in Am-20 and the maximum in Am-3. Present results are comparable with those of Tetarwal *et al.* (2008) who found that the conidiophore length and breadth among six isolates of *Alternaria alternata* varying from 18.90-27.40 and 4.23-5.75 µm. Studies on conidiophore septation revealed the presence of aseptate to septate conidiophore. Septate as well as aseptate conidiophores were observed in Am-1, Am-5, Am-9, Am-14, Am-19 and Am-21, while in remaining 15 isolates only septate conidiophores were observed. The overall highest septation of 9 was recorded in Am-4. Maximum mean septation (5.90) was recorded in Am-5, while the least mean septation was in Am-2 (3.08). The conidial septation

both transverse and longitudinal varied significantly among the isolates. Transverse septation varied from 1-7 and longitudinal from 0-4. The highest mean number of transverse septa (2.95) was recorded in Am-6 while the lowest (1.76) was in Am-8. The highest mean number of longitudinal septa (1.12) was recorded in Am-1 while the lowest (0.22) was in Am-14. Conidia with no longitudinal septa were observed in all the isolates tested. In the present study, significant variation in conidial size was noticed among the 21 isolates. The average conidial size varied from 21.36-31.74 x 8.34-14.48 µm. The average maximum (31.74 µm) and minimum (21.36 µm) conidial length were recorded in Am-13 and Am-16, respectively, while average maximum (14.48 µm) and minimum (8.34 µm) conidial breadth were recorded in Am-9 and Am-16, respectively. Similar observations were also made by Rotem (1966) who found a wide range of variability in the spore dimensions of forty two isolates of *A. solani*. Varma *et al.* (2006) reported that the average conidial size of *Alternaria solani* varied from 150.0-224.9 x 12.4-17.2 µm, they also reported that the horizontal septation varied from 4-14 and vertical from 0-3. Similarly Rai and Kumari (2009) reported variation in conidial length and breadth in isolates of *Alternaria alternata* with length varying between 25.80 and 48.22 µm and breadth between 8.13 and 23.24 µm. They also found number of horizontal and vertical septa varying between 3 to 8 and 2 to 5

respectively. Quayyum *et al*. (2005) reported significant variation in conidial length of *Alternaria panax* isolates from American ginseng, but found non-significant variation in their width.

Isolates of *A. mali* varied little in their colour of hyphae, conidiophore and conidia as well, which was mostly around olive. Colours of the hyphae and conidiophore were nearly hyaline, light olive green, olive green or dark olive. Conidial colour of four isolates was light olive green, six isolates olive green, six isolates dark olive and five isolates dark olive green. In similar studies little variation in conidial colour of *Alternaria panax* was observed by Quayyum *et al*. (2005). Shape of conidia was another criterion studied for variation among the isolates. All the isolates had muriform and obclavate conidia, with slight differences in their shape. Nine isolates had ovate to round, six isolates had oval to ellipsoidal and six had pyriform shaped conidia. Similar observations were made by Mortensen and Bergman (1983) who found slight differences in morphology of conidia among 35 isolates of *Alternaria carthami*. Quayyum *et al*. (2005) also observed little variation in conidial shape of *Alternaria panax*.

The present study indicated the prevalence of high pathogenic variability among the isolates. All the isolates were successful in producing

the disease lesions with variation in their incubation period, number of lesions and size of lesions. The incubation period of isolates varied from 2.0 to 6.3 days with minimum of 2.0 days for Am-9, Am-13 and Am-15, and a maximum of 6.3 days in Am-17. The number of lesions produced by the isolates varied from 6.3 to 14.3 with the least produced by Am-2 and maximum by Am-16. The mean minimum lesion size of 2.9 mm was recorded in Am-17 while the maximum of 10.2 mm was observed in Am-1. Our findings are in agreement with Thrall *et al.* (2005) who found significant differences in the lesion size produced by the *Alternaria brassicicola* isolates on *Cakile maritima*, when plants were wound inoculated. Kumar (2004) also reported variation in lesion size and lesion number produced by the isolates of *Alternaria triticina*. Among the two isolates of *Alternaria carthami* one produced more severe leaf spots than other when inoculated on safflower cultivars (Rai and Kumari, 2009). However 5the observations are contradictory to the findings of Quayyum *et al*. (2005) who did not find any significant variation in the lesions produced by the isolates of *Alternaria panax* on detached leaflets of ginseng. The isolate Am-1 was highly virulent as compared to other isolates with the incubation period of 2.6 days, producing 12 lesions of 10.2 mm average size. Filajdic and Sutton (1992) tested eight isolates of *A. mali* from Western north Carolina for pathogenicity and virulence, and found all the

isolates pathogenic on delicious seedlings with varied virulence. Varma *et al.* (2006) reported variability in virulence among isolates of *Alternaria solani*. In contrast Van der Waals *et al.* (2004) found a moderate degree of variation in virulence among different isolates of *Alternaria solani*.

The dendrogram analysis of the isolates on the basis of cultural, morphological and pathogenic studies revealed high diversity within *A. mali* population. Several workers have reported cultural, morphological and pathogenic variability among isolates of *Alternaria* spp. (Saharan and Kadian, 1983; Khan *et al*., 1992; Vishwanath and Kolte, 1997; Pandey and Vishwakarma, 1999; Singh *et al*., 2003; Slavov *et al*., 2004 and Tetarwal *et al*., 2008). At 67 per cent similarity matrix, all the isolates were categorized into 2 (I and II) clusters. Cluster I contained 12 isolates, and cluster II accommodated 9 isolates. Cluster I was further subdivided into Ia and Ib groups accommodating 7 and 5 isolates, respectively. Similarly, cluster II was also subdivided into IIa and IIb accommodating 6 and 3 isolates, respectively. The similarity among the isolates ranged from 57.6 to 82.1 per cent with the minimum between Am-10 and Am-21 and the maximum between Am-2 and Am-10 and Am-4 and Am-11. It can be argued that variation in the isolates may be inherent since isolates were collected from different sites. Phenotypic characters are influenced by environmental

conditions so may be responsible for such diversity. Moreover isolates in these sites may have adapted for many years which may be responsible for this diversity. Many of the phenotypic markers being controlled by many genes most of which have additive effect cannot be ruled out. The tendency to group isolates in relation to their geographical location was not obtained, as the two major clusters (I and II) as well as the sub-clusters (Ia, Ib and IIa, IIb) contained isolates from different districts. Brierley (1920) suggested that variation in fungi imperfecti may be due to mutation, or by splitting of an originally impure genetic constitution or of gametic or somatic segregation from heterozygotes. Variants, considered as due to mutation, were found in single spore cultures of *A. mali* (Roberts, 1924).

DNA based molecular markers that are selectively neutral and randomly distributed in a genome can be important tool to study genetic diversity of pathogen populations (Dini-Andreote *et al*., 2009). In absence of defined differential set availability, molecular markers offer an appropriate alternative to detect and characterize genetic variability in *Alternaria mali*. RAPD analysis is extremely robust and can separate individuals having intra- and inter-specific variability. It gives more comprehensive information regarding the genetic variability among the pathogen populations as is based on the entire genome of an organism

(Achenback *et al*., 1997). For molecular characterization of *Alternaria mali* isolates with RAPD markers, one 10-mer (OPA-13) and three 20-mers primers *viz.* U2, U3 and U9 producing consistent polymorphic bands were selected for fingerprinting. Kumar *et al*. (2008) selected four random primers to study the genetic diversity within isolates of *A. solani* because of their reproducible results of polymorphism between individuals. In the present study, highest number of scored bands was 14 in U3 and all the bands were polymorphic thus showing 100 per cent polymorphism, while as lowest number of scorable bands (7) were observed with U2 of which 6 bands were polymorphic. In total 42 bands were scored of which 41 were polymorphic thereby showing 97.61 per cent polymorphism. The dendrogram analysis of 21 isolates revealed a high genetic diversity within *A. mali* population in Kashmir valley. At 57.5 per cent Dice similarity coefficient all the isolates form a single clade, thus showing 42.50 per cent dissimilarity among the isolates. The results presented are in agreement with the observation of Guo *et al*. (2004), who found high genetic variation within *A. alternata* isolates originating from *Pinus tabulaeformis* and reported that *A. alternata* appears to have the potential for relatively quick evolution, which may lead to significant diversification. The results presented do not agree with observation of Pusz (2009) who reported low genetic variability among the isolates of *Alternaria alternata* originating

from *Amaranthus* however, he stated that the low genetic variation may be the result of high adaptive ability of the fungus and of the close proximity between collection sites and host-plants. At 68 per cent Dice similarity coefficient, all the isolates were categorized into 5 clusters (I to V). Cluster I was further subdivided into Ia and Ib groups accommodating 9 and 2 isolates, respectively. The tendency to group isolates in relation to their location proximity was not obtained, as the 5 clusters (I to V) and sub-clusters (Ia and Ib) contained isolates from different districts. This is in agreement with Varma *et al.* (2006) who reported no evidence for geographical clustering of *Alternaria solani* isolates with high level of genetic similarity suggesting that isolates were widely spread across India. Kumar *et al.* (2008) also found no effect of origin of isolation, rather two isolates of *Alternaria solani* from two different locations were closer to each other. Van der Waals *et al.* (2004) has shown high genetic diversity among *A. solani* isolates from South Africa and found no clustering of isolates according to geographical origin. Dice Similarity percentage of the isolates ranged from 38.0-91.2 with the minimum between Am-5 and Am-7 and maximum between Am-1 and Am-21. The RAPD data presented here and other molecular studies (Adachi *et al*., 1993; Weir *et al*., 1998; Morris *et al*., 2000; Roberts *et al*., 2000; Bock *et al*., 2002; Tigano *et al*., 2003) confirm high level of variation among isolates of a single species of

Alternaria. A possible explanation for the high level of genetic diversity found among isolates of *A. mali* could be natural chance mutations, combined with the fact that the fungus can produce abundant numbers of spores in a relatively short period of time as reported in *Alternaria alternata* (Petrunak and Christ, 1992; Leung *et al.* 1993). However, high levels of genetic variations are usually due to recombination, which occurs sexually through mating or asexually through the parasexual cycle. It is not known if the parasexual cycle occurs regularly in the genus *Alternaria*, and there is no known sexual cycle for *A. mali* (Simmons, 1986; Petrunak and Christ, 1992; Cooke *et al*. 1998). Morris *et al*. (2000) suggested an unknown sexual stage as a possible explanation for this diversity as *Alternaria* anamorphs of several *Lewia* spp. have already been described (Simmons, 1986). The high diversity in Kashmir valley may be because of the mixed cultivars in our orchard eco-system, diversity in sites and selection pressure due to indiscriminate use of fungicides. Slavov *et al*. (2004) has argued the opportunities for genetic change through mutation, nuclear migration and anastomoses whereby any mycelium may become heterokaryotic is more likely in *Alternaria*. Obviously the conidia produced from such mycelium will be genetically different. RAPD analysis proved an efficient method for detecting genetic variability of *A. mali* isolates occurring in apple. However, additional work is needed to be carried out in *A. mali* populations

which includes evaluation of larger sample sizes and isolates from more diverse locations.

There was no congruence between the RAPD pattern and cultural, morphological and pathogenic characters. Isolates identical for one spectrum were most often dissimilar for other spectrum. The findings are supported by Kumar *et al*. (2008), who reported that groupings based on RAPD data could not be correlated to the ones based on cultural, morphology and pathogenicity. Peever *et al*. (2002) also did not find any correlation between RAPD clustering of the *Alternaria* isolates and pathogenicity.

The present variability study based on cultural, morphological, pathogenic and molecular characters revealed no grouping of isolates having proximity of the locations. These results indicate the presence of mixed sub-populations, which may be due to the distribution of vegetative material over the entire valley (across districts and locations), as the disease is prevalent in apple seedlings as well. Another possible cause for even distribution of genetic variation in *A. mali* may be the dissemination of spores by biotic and abiotic factors. The distribution of genetic variation of *A. mali* gives an indication of the fitness of the pathogen to circumvent the effects of natural or artificial stresses on the population, and thus counteract

control measures such as fungicide applications (McDonald *et al.,* 1989; Adachi *et al.,* 1993; McDonald and Linde, 2002). McDonald and Linde (2002) have suggested that in such cases breeding efforts should concentrate on quantitative resistance or on the development of cultivar mixtures that can be used in combination with other control strategies. Understanding the diversity of *A. mali* on apple in Kashmir will thus aid in disease management strategies of Alternaria leaf blotch.

Variation in cultural, morphological, pathogenic and molecular characters of isolates observed indicated the existence of different strains of pathogen. Similar characters have formed the basis for defining the existence of different strains among species of the fungi imperfecti (Shear and Wood, 1913; Crabill, 1915; Brierley, 1920; Burger, 1921; Boner, 1922; Stevens, 1922). Various other authors have indicated the existence of races of *A. solani* based on morphological, physiological, and virulence differences (Bonde, 1929; Henning and Alexander, 1959; Neergaard, 1945). As Stackman *et al.* (1981) pointed out biological forms as threat in case of stem rust of wheat, similarly biological forms of *Alternaria mali* may pose a problem to breeding for resistance to Alternaria leaf blotch of apple.

Exploiting the inherent resistance of crop plants to pathogenic infection is an economical, eco-friendly and easy disease management

venture. All the 13 apple cultivars screened against most virulent isolate of *A. mali* succumbed to the leaf blotch with varied intensities. Pooled data revealed highest disease intensity of 28.36 per cent in Red Delicious followed by 22.04 per cent in Red Gold. In American Apirouge disease intensity of 3.92 per cent was recorded while White Dotted Red was least effected with disease intensity of 0.66 per cent. Attempts to identify the apple cultivars showing resistance to *A. mali* under natural conditions indicated only White Dotted Red to be highly resistant, while American Apirouge showed resistant reaction. Three cultivars *viz*., Firdous, Shireen and Sparten were moderately resistant and two cultivars *viz*., Red Delicious and Red Gold were highly susceptible. These findings are in agreement with Bhat (2002) who reported various bio-chemical constituents such as low sugar, high phenol, low phenol oxidase and high peroxidase activity as contributing factor towards the resistance of White Dotted Red to Alternaria leaf blotch. Filajdic and Sutton (1991) also reported cultivars of Delicious more susceptible to *Alternaria mali*. Shahzad (2003) reported White Dotted Red as tolerant and Red Delicious as highly susceptible. Shahzad (2003) screened 22 cultivars under natural conditions while in present study 13 cultivars were screened against the most virulent isolate Am-1. It is expected that Alternaria leaf blotch will increase in future because susceptible cultivars of Delicious are commonly grown in Kashmir valley.

The use of SAR (systemic acquired resistance) activators for disease control is safe application because SAR activators neither have any toxic effect on pathogens, plants and animals, nor show any inhibitory effect on plant growth, development and yield. Besides SAR activators show broad spectrum of defense, require low loading amount, and provide long lasting protection to plants (Kessman *et al*., 1994; Tally *et al*., 1999; Kuc, 2001). SAR chemicals in present study significantly lowered disease intensity as compared to check. The disease intensity ranged from 5.88 to 17.34 per cent in SAR treatments in comparison to 35.35 per cent in check. The least disease intensity of 5.88 per cent was observed in BABA (β-aminobutyric acid) which was at par with fungicide Penconazole treatment showing disease intensity of 5.56 per cent. Both the treatments were however, significantly superior to other chemicals evaluated. These findings are in agreement with Agostini *et al*. (2003) and Percival and Haynes (2008) who reported SAR activators to be generally less effective than standard synthetic fungicides in the control of foliar pathogens. Percival *et al.* (2009) reported penconazole superior over Messenger (a.i. Harpin protein), Phoenix (a.i. Potassium phosphite) and Rigel (a.i. Salicylic acid derivative) in controlling apple and pear scab. However, Tally *et al.* (1999) found BTH more efficient with 90 per cent disease reduction on the 17^{th} day after its application as against only 46 per cent for metalaxyl.

They also reported BTH as an effective inducer of resistance in tobacco against fungal pathogens, viruses and bacteria. The other SAR chemicals in decreasing order of their efficacy were K_3PO_4, BTH, INA, $Ca(OH)_2$, K_2HPO_4 and $CaCO_3$. Gottstein and Kuc (1989) suggests phosphate application for disease control because of the effectiveness of systemic resistance induced by phosphates, their low cost, low animal toxicity, nutrient value and comparative safety for the environment. Present results regarding INA are in agreement with the findings of Kessmann *et al.* (1994) who reported that the foliar spray of INA reduced fire blight (*Erwinia carotovora*) by 45 per cent compared to non inoculated control. Calcium based chemicals used in our study were effective in combating the disease. Our results are in agreement with the findings of Yoon *et al.* (1989) who reported effectiveness of application of $Ca(OH)_2$ and $CaCO_3$ against *Alternaria mali* and also found higher calcium content in apple leaves responsible for imparting resistance to *Alternaria mali* than in the leaves of susceptible ones. Application of SAR activators proved significantly superior when used before pathogen application as against post inoculation application. Our observations are in accordance with the findings of numerous workers (Schonbeck *et al*., 1993; Kessman *et al*., 1994; Schneider *et al*., 1996; Van Loon *et al*., 1998; Benhamou and Picard, 1999; Tally *et al*., 1999; Cohen, 2001; Kuc, 2001; Bokshi *et al*., 2003; Gozzo,

2003; Soylu *et al*., 2003). Yoon *et al*. (1989) reported that the application of calcium compounds to apple leaves prior to inoculation of *Alternaria mali* is more effective than after inoculation. In present study application of BABA before and after inoculation of the *Alternaria mali* proved superior to all other SAR chemicals tested. Similar observations have been recorded by Amzalek and Cohen (2007) who found foliar application of BABA provided significant protection against sunflower rust, while BTH and INA were less protective. The post infection inhibitory effect of BABA better than BTH is because the BABA operates quite rapidly to stop growth of mycelia in the mesophyll as reported by Amzalek and Cohen (2007). Reuveni *et al*. (2003) reported control of moldy-core in apple by DL-β-aminobutyric acids (BABA) and potassium phosphite in the laboratory and in the field. They showed that three foliar applications of BABA or potassium phosphite, starting from the initial bloom to petal fall, reduced the number of fruits infected with moldy-core by 40 and 60%, respectively, in comparison to untreated control. Chemically-induced SAR has also been reported effective against *Peronospora tabacina*, *Cercospora nicotianae*, *Phytophthora parasitica* var. *nicotianae*, *Pseudomonas syringae* pv. *tabaci* and TMV (Lyon *et al*.,1995; Strobel and Kuc, 1995; Schneider *et al*., 1996; Kuc, 2001).

CHAPTER - 6

SUMMARY AND CONCLUSION

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The investigations on Alternaria leaf blotch of apple in Kashmir valley were carried out during the year 2007 to 2009. The results of the study are summarized as under:

The survey of apple orchards in seven districts of Kashmir valley *viz*. Bandipora, Baramulla, Budgam, Ganderbal, Kulgam, Pulwama and Shopian, revealed the prevalence of the disease in all the seven districts of Kashmir valley with an overall incidence and intensity of 42.11 and 21.68 per cent, respectively. The highest disease incidence (51.94%) and intensity (26.34%) was observed in district Pulwama and least in district Shopian (25.49 and 12.52% respectively). Among the locations surveyed, Batwina in district Ganderbal exhibited highest disease incidence (68.74%) and intensity (33.62%) while least disease incidence and intensity of 19.33 and 9.21 per cent was recorded in Kachdoora of district Shopian.

The causal organism involved for leaf blotch (*Alternaria mali*) was isolated from diseased leaves of apple cv. Red Delicious and subsequently maintained for further studies. The pathogenicity of twenty one isolates from various locations was established following Koch's postulates.

Variations in pathogen population were studied on the basis of their cultural, morphological and pathogenic behaviour. Also, molecular technique was adopted to record any genetic variation among the isolates. Cultural characteristics studied were colony type, colour, margin, pigmentation and growth rate. Colonies grew slowly exhibiting velvety or cottony growth and were mostly appressed. The colony colour ranged from light olivacious to dark olivacious with regular to irregular margins. Isolates varied in their growth rates however; all the isolates depicted an overall increasing trend in growth rate from 2nd day to eight day and decreased afterwards. Am-13 with mean growth rate of 8.21 mm was fastest while Am-5 with mean growth rate of 5.86 mm was slowest. Isolates also depicted periodic changes in their growth rates. After 2, 4, 6, 8 and 10 days of inoculation highest growth rate of 5.25, 10.75, 13.00, 18.35 and 5.58 mm were recorded in Am-3, Am-13, Am-7, Am-14 and Am-9, respectively and least in Am-18 (1.08 mm), Am-19 (4.16 mm), Am-14 (6.50 mm), Am-5 (9.75 mm) and Am-11 (0.16 mm), respectively. All the isolates impregnated the media with a colour pigment mostly grey to brown and were clearly visible from under side of the plates.

Morphologically isolates varied in size, colour, shape and septation of their hyphae, conidiophore and conidia. Mean hyphal width ranged from

3.28 to 4.28 µm with maximum in Am-4 and minimum in Am-21. Significant variation was observed in the length and breadth of conidiophore with maximum length (68.18 mm) in Am-9 and the minimum (26.99 mm) in Am-17. Conidiophore breadth varied from 3.30 to 4.70 µm with least in Am-20 and maximum in Am-3. Conidiophores were septate as well as aseptate and maximum mean septation of 5.90 was recorded in Am-5 and least of 3.08 in Am-2. Aseptate conidiophores were observed in Am-1, Am-5, Am-9, Am-14, Am-19 and Am-21 and highest septation of 9 in Am-4. Conidial septation, both transverse and longitudinal, showed significant variation among the isolates. Transverse septation varied from 1-7 and longitudinal from 0-4. The highest mean number of transverse septa (2.95) was in Am-6 while lowest (1.76) in Am-8. Similarly highest mean number of longitudinal septa (1.12) was in Am-1 while lowest (0.22) in Am-14. Minimum of one transverse septum was in all the isolates while the maximum of seven was in Am-18. Conidia without longitudinal septa were observed in all the isolates tested while the upper range was 3 to 4. The average conidial size of isolates varied from 21.36-31.74 µm x 8.34-14.48 µm with maximum (31.74 µm) and minimum (21.36 µm) conidial length in Am-13 and Am-16, respectively. The average maximum and minimum conidial breadth of 14.48 µm and 8.34 µm was in Am-9 and Am-16, respectively.

Isolates of *A. mali* varied in the colour of hyphae, conidiophore and conidia. Hyphal and conidiophore colour varied from nearly hyaline, light olive green to dark olive. While as conidia of four isolates were light olive green, six were olive green, six dark olives and five isolates dark olive green. All the isolates had muriform and obclavate conidia and variations were in their being either ovate to round, oval to ellipsoidal or pyriform. Nine isolates were ovate to round, six oval to ellipsoidal and six with pyriform shaped conidia.

Studies indicated the prevalence of high pathogenic variability among the isolates. All the isolates successfully produced disease lesions however, varied in their incubation period and number and size of lesions. Incubation period varied from 2.0 to 6.3 days with minimum in Am-9, Am-13 and Am-15 and maximum in Am-17. The number of lesions produced by isolates varied from 6.3 to 14.3. Least number of lesions was produced by Am-2 and maximum by Am-16. The mean minimum lesion size of 2.9 mm was recorded in Am-17 while the maximum of 10.2 mm was observed in Am-1. The isolate Am-1 was highly virulent with an average lesion size of 10.2 mm.

Data generated from the cultural, morphological and pathogenic studies was used to construct a dendrogram to determine distance/similarity

between isolates. The dendrogram analysis revealed high diversity within *A. mali* population. All the isolates were within single group at 66 per cent similarity coefficient, while as at 67 per cent similarity coefficient the isolates were categorized into 2 clusters (I and II). Cluster I had 12 isolates, while as cluster II accommodated 9 isolates. The similarity among the isolates ranged from 57.6 to 82.1 per cent with minimum between Am-10 and Am-21 and the maximum between Am-2 and Am-10 as well as Am-4 and Am-11.

For molecular characterization, RAPD markers [one 10-mer (OPA-13) and three 20-mers *viz.*, U2, U3 and U9] producing consistent polymorphic bands were selected for fingerprinting of isolates. In present study 42 bands were scored of which 41 were polymorphic thus showing 97.61 per cent polymorphism. The dendrogram analysis revealed a high genetic diversity within *A. mali* population in Kashmir valley. At 68 per cent Dice similarity coefficient, all the isolates were categorized into 5 clusters (I to V) however, above 91.2 per cent Dice similarity coefficient all the isolates were totally dissimilar. Tendency to group isolates in relation to their location proximity was not obtained. Dice similarity percentage of isolates ranged from 38.0 to 91.2 with minimum between Am-5 and Am-7 and maximum between Am-1 and Am-21. There was no congruence

between RAPD pattern and cultural, morphological and pathogenic characters. Isolates that were identical for one spectrum were most often dissimilar for other spectrum.

Of the thirteen apple cultivars screened for resistance to Alternaria leaf blotch, only one cultivar 'White Dotted Red' showed highly resistant reaction followed by 'American Apirouge'. Three cultivars Firdous, Shireen and Sparten were moderately resistant whereas two cultivars, Red Delicious and Red Gold were highly susceptible.

Seven SAR (systemic acquired resistance) activators were evaluated against Alternaria leaf blotch disease. The disease intensity ranged from 5.88 to 17.34 per cent in chemical (SAR activators) treatments in comparison to 35.35 per cent in check indicating that all the SAR chemicals were significantly effective in lowering the disease intensity. Chemical application before pathogenic inoculation proved superior to the chemical application after pathogenic inoculation. BABA at 2000 ppm was highly effective before as well as after pathogenic inoculation with least disease intensity of 1.47 and 4.03 per cent, respectively; and was the only chemical at par with penconazole (standard), though other SAR activators were statistically inferior to penconazole at 400 and 500 ppm. The other SAR chemicals in decreasing order of their efficacy were K_3PO_4, BTH, INA,

$Ca(OH)_2$, K_2HPO_4 and $CaCO_3$.

From the present study, it is concluded that:

- Alternaria leaf blotch of apple is prevalent in almost all apple growing areas of Kashmir valley in moderate to severe form so is a potential threat to existing apple plantation. *Alternaria mali* isolates exhibited considerable variability in their morphological, cultural, pathogenic and genetic characters. The grouping based on RAPD data could not be correlated with grouping based on morphological, cultural and pathogenic characters. The wide variation of isolates indicated the possible existence of different strains of pathogen and indicates that the fungus has a high potential to adapt to resistant cultivars or fungicides. The evidence of strain existence and random distribution of variability in pathogen population have practical implications for breeding programs as well as in the management of Alternaria leaf blotch. Natural resistance of varying degree was observed in available apple germplasm which may be exploited in future. SAR compounds, especially BABA were effective in managing the disease. This induced resistance exploiting natural defense machinery of plants could be proposed as an alternative, non-conventional and eco-friendly approach for plant protection.

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LITERATURE CITED

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*Original not seen.

APPENDIX – I

Monthly meteorological data of Kashmir Valley for the year 2007 and 2008

Month	Relative humidity (%)		Rainfall (mm)	
	2007	**2008**	**2007**	**2008**
May	64.7	70.1	70.7	89.3
June	65.7	73.0	67.0	88.8
July	70.8	74.0	68.8	66.0
August	71.2	75.9	63.7	81.8
Mean	**68.1**	**73.2**	**67.5**	**81.5**

Source: Indian Meteorological Department, Meteorological Centre, Rambagh, Srinagar-190015.

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CERTIFICATE

Certified that all the corrections and modifications suggested by External Examiner (s) have been taken care of in the thesis manuscript entitled "**Variability in *Alternaria mali* (Roberts) and Management of Alternaria Leaf Blotch of Apple through Systemic Acquired Resistance Activators"** submitted by **Tariq Ahmad Sofi (Registration No. 2006-154-D)** before final binding.

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