PATHOGENIC VARIABILITY IN SORGHUM ANTHRACNOSE INCITED BY *Colletotrichum graminicola* (Ces.) Wilson

By

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B.Sc (Ag.)

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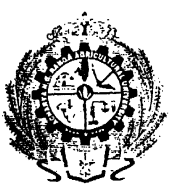

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CERTIFICATE

Mr.SHUVENDU HAZRA has satisfactorily prosecuted the course of research and that the thesis entitled **PATHOGENIC VARIABILITY IN SORGHUM ANTHRACNOSE INCITED BY *Colletotrichum graminicola* (Ces.) Wilson** submitted is the result of original research work and is of sufficiently high standard to warrant its presentation to the examination. I also certify that the thesis or part thereof has not been previously submitted by him for a degree of any University.

Date : 30.12.05

Place: Hyderabad

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Major Advisor

CERTIFICATE

This is to certify that the thesis entitled **PATHOGENIC VARIABILITY IN SORGHUM ANTHRACNOSE INCITED BY *Colletotrichum graminicola* (Ces.) Wilson** submitted in partial fulfilment of the requirements for the degree of **MASTER OF SCIENCE IN AGRICULTURE** of the Acharya N.G.Ranga Agricultural University, Hyderabad, is a record of the bonafide research work carried out by **Mr.SHUVENDU HAZRA** under my guidance and supervision. The subject of the thesis has been approved by the student's Advisory Committee.

No part of the thesis has been submitted for any other degree or diploma or has been published. The published part has been fully acknowledged. All the assistance and help received during the course of investigations have been duly acknowledged by the author of the thesis.

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LIST OF ABBREVIATIONS

C	-	Celsius
cm	-	centimeter
Fig.	-	Figure
g	-	Gram
ha	-	hectare
hrs	-	hours
ISAVN	-	International Sorghum Anthracnose Virulence Nursery
kg	-	kilogram
L	-	litre
LSD	-	Least significant difference
ml	-	millilitre
mm	-	millimetre
mM	-	Millimolar
nm	-	Nonometre
ng	-	Nanogram
No.	-	number
OMA	-	Oat meal agar
PSI	-	Pound per square inch
SEM	-	Standard error of mean
µ	-	Micron
µm	-	Micrometre
µl	-	Microlitre
µg	-	Microgram
µM	-	Micromolar

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DECLARATION

I, **Mr.SHUVENDU HAZRA** hereby declare that the thesis entitled **PATHOGENIC VARIABILITY IN SORGHUM ANTHRACNOSE INCITED BY *Colletotrichum graminicola* (Ces.) Wilson** submitted to Acharya N.G.Ranga Agricultural University for the degree of **MASTER OF SCIENCE IN AGRICULTURE** is the result of the original work done by me. It is further declared that the thesis or any part thereof has not been published earlier in any manner.

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ABSTRACT

Anthracnose, caused by *Colletotrichum graminicola* (Ces.) Wilson is an important disease of sorghum (*Sorghum bicolor* (L.) Moench) throughout the world. In this study, 12 isolates of *C. graminicola* were characterised for pathogenic variability. These isolates were obtained from four major sorghum growing states of India i.e. Maharashtra (Cg 94), Karnataka (Cg 20 and Cg 118), Andhra Pradesh (Cg 75, Cg 130, Cg 138, Cg 226, Cg 226-3, Cg 227 and Cg 227-3) and Tamil Nadu (Cg 150 and Cg 158). Isolation were made from leaf lamina, midrib and grains. The isolates were also characterized for morphological, cultural and genetic diversity.

Morphologically isolates varied widely with respect to conidial shape and size, presence, absence and morphology of setae, presence of appressoria and sclerotia, shape and size of appressoria on oat meal agar (OMA) medium. Based on morphological traits the 12 isolates were divided into six groups, although the same isolates were not included in a particular group in all cases. Three of the 12 isolates (Cg 94, Cg 227 and Cg 227-3) produced only falcate conidia whereas others produced both falcate and spindle shaped conidia (conidial dimorphism). Septation of appressoria observed for the first time in Cg 20 and Cg 130 while only Cg 138 produced lobed appressoria. All the isolates produced setae 10 days after incubation, except Cg 118. All 11 isolates varied significantly for the number of setae per acervulus and size of the setae. Isolate Cg 150 produced a second type of setae besides normal one which produced conidia on it. Other than Cg 130, Cg 158 and Cg 227 all the isolates produced sclerotia 20 days after incubation.

Variation was also observed in colony colour, growth and sporulation of all the isolates. Some isolates showed good sporulation with less mycelial growth whereas others gave less sporulation with good mycelial growth in 10-day-old culture. Colony colour varied from greyish white to salmon orange.

Pathogenicity test with the isolates showed mixed reaction to IS 8354 and IS 3758 among six sorghum differential lines. The other 4 differentials were either resistant or susceptible to all the isolates. Based on differential reaction, isolates could be divided into 3 pathotype groups.

The latent period differed significantly. The shortest duration was found in case of the most aggressive isolate Cg 226 on IS 3758 and longest for the least aggressive isolate Cg 118 on IS 3089. Similarly, virulence index was highest for Cg 226 and lowest for Cg 130 and Cg 118. Difference in virulence index was found between parental and monoconidial cultures (Cg 226 and Cg 226-3) but not in Cg 227 and Cg 227-3. Based on aggressiveness and virulence index the isolates were grouped into 3 and 4 clusters respectively.

Molecular study using Random Amplified Polymorphic DNA (RAPD) markers indicated more diversity than pathogenicity, morphological and cultural studies. Based on genetic similarity the isolates were grouped into four clusters of which Cg 138 was the most diverse one.

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INTRODUCTION

CHAPTER I

INTRODUCTION

Sorghum (*Sorghum bicolor* (L.) *Moench)* is an important cereal crop ranking fourth after rice, wheat and maize. It is grown as a staple food in semi-arid tropics and sub-tropics throughout the world. As a drought resistant crop it is adapted to a wide range of ecological conditions and produces reasonable grain yields under conditions unsuitable for most other cereals (Rosewich, 1996). The total area under sorghum cultivation is about 45.4 million hectares with a production of 64.2 million ton. India imparts the maximum area of 11.2 million ha producing 9.0 million ton grain yield (FAO, 1998). In India Maharashtra, Karnataka, Madhya Pradesh, Andhra Pradesh, Rajasthan, Gujrat, Tamil Nadu and Uttar Pradesh, produce more than 90 per cent of total production (Anahosur, 1992).

Although the crop is hardy enough, it still suffers from various biotic and abiotic stresses. Sorghum anthracnose (also known as red leaf blight and seedling blight) caused by *Colletotrichum graminicola* (Ces.) Wilson (=*Colletotrichum sublineolum* Henn. Kabat et Bub.) one of the main yield reducing factors. The disease is of great economic importance particularly under hot and humid conditions in most tropical and subtropical regions of the world, including India.

The fungus is a highly variable pathogen (Ali and Warren, 1992). The cultural and morphological variabilities of the pathogen create a confusion in identification on a specific host. Further more, phenotypic plasticity of the characters in artificial culture vary greatly and no

standardized cultural condition is followed by taxonomists. Also, it has been difficult to rationalize behaviours in culture with those on natural substrata. Identification of the pathogen has been broadly based on a combination of classical characters, such as conidial shape and size, presence, absence and morphology of setae, presence of sclerotia and appressoria and symptoms expression on the hosts (Sutton, 1992). Although the pathogen from maize was designated as *Colletotrichum graminicola* and from sorghum as *C. sublineolum* on the basis of their host specificity and cultural and morphological characters (Sutton, 1980) the controversy still exists from some other reports (Ali and Warren, 1992; Sherriff *et al.*, 1995; Rosewich, 1996). However, based on rDNA sequence analysis Sherriff *et al.* (1995) confirmed the distinction between *C.graminicola* and *C.sublineolum.*

The observations of International Sorghum Anthracnose Virulence Nursery (ISAVN) has revealed the difference in virulence within *C. graminicola* populations from different regions (King *et al.*, 1976; Thakur, 1995). Breakdown of resistance of sorghum cultivars also indicated the emergence of new pathotypes (Cardwell *et al.*, 1987; Gorbet, 1987) and later number of physiological races/ pathotypes have been reported from several parts of the world (Ferreira and Casela, 1986; Ali and Warren, 1987; Pande *et al.*, 1991). So the best strategy to control sorghum anthracnose is through the use of resistant varieties. Informations on the distribution of races/pathotypes and the extent and nature of variabilities are desirable because pathogenic variability increases the difficulty of breeding for disease resistance and deployment of sorghum cultivars (Guthrie *et al.*, 1992). For detection of races host differentials are used

assuming that such cultivars possess a vertical resistance phenotype (Frederiksen *et al.*, 1987). But this is a time consuming process so recently it has been amended by studies using Random Amplified Polymorphic DNA (RAPD) as electrophoretic markers (Casela, 1992; Guthrie *et al.*, 1992).

In India the existence of cultural, morphological and pathogenic variability in *C. graminicola* have been reported among the isolates from different states (Rao *et al.*, 1998; Mathur *et al.*, 1998). However, a clear picture about the pathotypes of four major sorghum growing states with special reference to Andhra Pradesh and efficiency of different methods to detect the pathotypes were not well known. Therefore, the present study was undertaken to determine the :

1. Diversity in morphology in artificial culture.
2. Diversity in cultural characteristics on oat meal agar medium.
3. Pathogenic variability using host differentials.
4. DNA polymorphism using random amplified polymorphic DNA (RAPD) markers.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

The literature reviewed in this dissertation is grouped into the following sections.

1. General
2. Morphological variability
3. Cultural variability
4. Pathogenic variability
5. Molecular variability

2.1 GENERAL

2.1.1 Distribution

Sorghum anthracnose, caused by *Colletotrichum graminicola* (Ces.) Wilson, has been reported from most areas where sorghum is grown, but is more prevalent and severe in warm and humid regions (Porter, 1962; Sundaram *et al.*, 1972; Bergquist, 1973). The disease was first reported from Togo, West Africa in 1902 (Sutton, 1980) though it had been known for sometime in India and elsewhere (Tarr, 1962). Since then the disease was reported from several countries of the world including Australia (Noble, 1937), USA (Miller, 1956), Mexico (Hsi, 1956), East Asia (Porter, 1962), India (Sundaram *et al.*, 1972), Hawaii (Bergquist, 1973) and Brazil (Pastor-Corrales and Frederiksen, 1979).

In India, the disease occurs mainly in parts of Karnataka, Tamil Nadu, Maharashtra, and Andhra Pradesh. It also occurs in sporadic form in Madhya Pradesh and Uttar Pradesh (Anahosur, 1992), Rajastan and Gujrat (Rao *et al.*, 1998).

2.1.2 Economic importance

Several workers have done extensive studies on grain loss, disease incidence and their relationship.

Losses in grain yield was estimated to exceed 50 per cent on susceptible sorghum cultivars in a severe epiphytotic in Georgia (Harris *et al.*, 1964). In Guatemala and Venezuela it was one of the main yield limiting factors (Maunder, 1975). Powell *et al.* (1977) reported that the grain yield was reduced by 70 per cent and more than half the yield loss resulted due to incomplete grain filling. Gorbet (1987) reported that grain production of susceptible sorghum cultivars is severely limited when the disease develops during heading or early grain filling.

Anthracnose is the most important disease in Brazil and is a major threat in most of the Latin American countries (Pastor-Corrales and Frederiksen, 1980). Neya and Kabore (1987) reported that infection by *C. graminicola* resulted in 46 per cent loss in yield on a local susceptible cultivar in Burkino Faso (West Africa).

In India, the percentage loss in sorghum grain yield varied from 1.2 to 16.4 depending upon anthracnose severity (Mishra and Siradhana, 1979). Luttrel (1950) reported that serious yield losses may not occur if

leaf symptoms do not appear until after the plant mature. Sharma (1980) also observed that losses due to anthracnose generally ranged between 41 per cent and 60 per cent. However,Chohan (1967) reported that infected grains lose 51 per cent in weight as compared with healthy grains from plants of the same age. Ferreira and Warren (1982) estimated grain losses caused by anthracnose to reach as high as 88.7 per cent in the highly susceptible cultivar.

From their study, Basuchowdhary and Mathur (1979) reported 55 per cent seed rot and 45 per cent seedling mortality in infected samples whereas Saifulla and Ranganathaiah (1989) reported upto 23 per cent seed infection in susceptible cultivars.

2.1.3 Symptoms

The characteristic symptoms caused by *C. graminicola* in sorghum have been adequately described (Tarr, 1962; Pastor-Corrales and Frederiksen, 1980; Dickson, 1956; Williams *et al.*, 1978). It infects leaves, stalks, peduncles, panicle and the grain either separately or all together (Pastor-Corrales and Frederiksen, 1980). Disease symptoms include a foliar phase, stalk rot, and panicle and grain anthracnose. Infection on the leaves can occur at all stages of plant growth. In this phase the disease produces small circular to elliptical spots upto about 6-7 mm diameter that develop on the leaves and leaf sheaths. They are well defined and colour ranges from tan, orange-red to blackish purple depending on the variety of the crop attacked. The centre of the old spots become greyish or straw coloured with reddish border and have few

or several minute black specks, the fruiting body (acervuli). Midrib infection generally occurs as elliptical discoloured areas which may coalesce to affect most of the midrib. Sometimes leaf spot may coalesce together causing extensive necrosis.

Symptoms of stalk rot can be recognized by irregularly mottled and marbled pattern of colonization. In the begining the interior of the stalk is water-soaked and discoloured but no external symptoms are visible. The colouration depends on variety of sorghum ranging from yellowish to blackish purple and appearing more or less uniform or mottled with whitish patches. Stalk rot is generally preceded by leaf anthracnose. The upper internodes and peduncles seem to be especially liable to invasion, the dry, rotted internode tissue shrinks and may lead to breaking of the stalks. However, stalk rot phase is not prevalent in India. On grains, symptoms can be seen as small black dots on its surface.

2.1.4 The causal organism

The anthracnose fungus has been described by a number of reviewers (Mordue, 1967; Pastor-Corrales and Frederiksen, 1980; Tarr, 1962; Warren, 1986). The imperfect stage of the fungus, *Colletotrichum graminicola* (Ces.) Wilson causes anthracnose of other cereals and many grasses as well. The name *C. graminicola* was created by Wilson (1914) to include most forms of *Colletotrichum* with falcate conidia. Synonyms encountered in the early literature include *Dicladium graminicola* introduced by Cesati in 1852, but there was no mention of the taxonomic relationship in the original account. The type species was then placed in *Vermicularia*

by Westendorp (1861) and later Saccardo (1886) placed it in *Steirochaete* Braun and Caspari, thinking that the conidia were catenate (Sutton, 1992). It was finally referred to *Colletotrichum* by Wilson (1914). However, after thorough morphological studies of isolates from maize and sorghum Sutton (1980) designated the isolates from maize being *C. graminicola* and those from sorghum as *C. sublineolum* considering the structure of appressoria they produced.

According to Jamil and Nicholson (1987) isolates from maize and sorghum showed strict host specificity, So the forms of *C. graminicola* that attack maize and those that attack sorghum are distinct being either *formae specialis* or possibly different species. Support for the distinction of these two species came from studies of isozymes (Huguenin *et al.*, 1982; Ali *et al.*, 1989) and from evidence of their genetic isolation and DNA fingerprints (Vaillancourt and Hanau, 1991, 1992; Sherriff *et al.*, 1995). But still *C. sublineolum* has not been widely accepted as a distinct species and is rarely referred in reviews or research papers (Sherriff *et al.*, 1995).

Politis and Wheeler (1972) first reported the production of teleomorphic stage of *Colletotrichum graminicola* from corn. Later Politis (1975) differentiated the perfect state of *C. graminicola* and *C. falcatum* by size of perithecia, asci and ascospores as well as on shape of ascospores. Accordingly the perfect state of *C. graminicola* was named as *Glomerella graminicola*.

2.1.5 Taxonomy of the pathogen

Members of the genus *Colletotrichum* Cda. belong to the subdivision Deuteromycotina - the imperfect fungi reproduced by mitotically produced spores. The subdivision been further splitted into two classes, whereby *Colletotrichum* has been systematized to belong to the Coelomycetes (conidia generated in pycnidial, pycnothyral, acervular, cupulate or stromatic conidiomata) and further as a member of the order Melanconiales (conidia produced in acervuli) (Hawksworth *et al.*, 1983).

As the perfect stage for *Colletotrichum* spp. is not commonly found in nature or has not even been described for certain species, most taxonomic approaches to establish relationship within these groups have relied on the morphology of the asexual stage. As mentioned by Sutton (1980), several hundred species of *Colletotrichum* were recognized at the beginning of the twentieth century. Von Arx (1957) reduced and reclassified the genus into 11 species. Sutton (1980) suggested 22 species of *Colletotrichum* should be differentiated. Due to aspects involving host specificity, this number has been increased to 39 species including some species with physiological taxa (Sutton, 1992). Wilson (1914) and Von Arx (1957) united most of the falcate spored *Colletotrichum* known from the Graminae into the group species *C. graminicola*. However molecular biology provides a new insight into systematics, particularly in the delimitation of species and defining inter and intraspecific relationship (Guthrie, 1993; Rosewich, 1996).

2.2 MORPHOLOGY

Mycelium of *Colletotrichum graminicola* is immersed, branched, septate, hyaline, pale brown or dark brown. It is characterized by conidiomata called acervulus which is present in the necrotic part of the lesion (Sutton, 1980). Acervuli on leaf and stem lesions appear as black rounded or elongated approximately 70-300 μm in diameter (Mordue, 1967). Occasional cells of the acervulus develop as setae which are brown, slightly swollen at the base, then tapered to the rounded often slightly paler tip on which conidia are sometimes formed. Conidiophores are numerous, short, crowded hyaline measuring 1-2 x 6-12 μm in size each of which bears a single terminal conidium.

2.2.1 Conidia

Differences in conidial morphology have been noted, but these attributed to irregular forms of falcate conidia (Mordue, 1967) whereas Sutton (1980) described them as falcate or lunate. Tarr (1962) has pointed out wide variation in conidial shape and dimension within *C. graminicola* which may possibly be associated with different strains of the pathogen. Host also plays an important role in conidial variation. Tiffany and Gilman (1954) compared the conidial shape and size of *C. graminicola* from oat and some leguminous plants and reported that size of the conidia not only differs from one host to another host but also differs between two isolates from same host collected from different places. Some host produced conidia with pointed end, while others produced rounded end conidia.

But he did not mention about the production of spindle shaped conidia by any host.

Mordue (1967) mentioned the range of conidial size but the size of conidia mentioned by various authors differ (Chowdhury, 1936; Sutton, 1980; Rajasab *et al.*, 1981). Considering the size of conidia and other morphological variations among isolates Rajasab *et al.* (1981) proposed a new *formae specialis* of the pathogen as *C. graminicola* var. *zonatum*. During their work with 9 isolates from different states of India, Pande *et al.* (1991) found the length of the conidia are significantly different among isolates and those 9 isolates were classified into five groups according to conidial length. Mathur *et al.* (1997) reported that size of the conidia varies significantly even among 12 monoconidial cultures of same isolate.

Lenne *et al*, (1984) observed that conidia produced from setal apices and these conidia are smaller than the conidia produced on conidiophores.

Nishihara (1975) reported the production of both oval and falcate conidia and concluded that oval conidia are more virulent than falcate conidia. Panaccione *et al.* (1989) confirmed this conidial dimorphism but could not find any difference in virulence. They concluded that falcate conidia disseminate the pathogen to new host and oval conidia may be responsible for distribution of the pathogen within host. [illegible] and Frederiksen (1995) worked on dynamics of oval and [illegible] conidia production and observed that in Frie's liquid media [illegible] dextrose broth maximum oval conidia produce within 2 to [illegible] inoculation.

2.2.2 Appressoria

Appressoria were first observed for *Fusicladium tremulae* Frank and *Gloeosporium lindemuthianum* sacc. and Magn. by Frank (1883). Later, Halstead (1893) found that *Colletotrichum* and *Gloeosporium* isolates from 24 different hosts produced appressoria. These specialized cells, formed before the penetration of host tissues, have since been demonstrated frequently in *Colletotrichum* and many other fungi. Hasselbring (1906) provided the first extensive study of appressoria formation and Simonds (1941), discussing the infection of tropical fruits by *Colletotrichum* reviewed the literature on their formation and relevance to latent infection.

Chowdhury (1936) reported that there is no significant difference between appressoria of *Colletotrichum graminicola* isolates from sorghum and maize. But Sutton (1968) observed that appressoria produced by maize isolates are more irregular in shape and larger in size than sorghum isolates. On this basis he regarded them as two species - the isolates from maize being *C. graminicola* and those from sorghum were designated as *C. sublineolum* (Sutton, 1980). He also suggested that appressorial characters may be used more extensively in the identification of *Colletotrichum* species (Sutton, 1962).

Appressoria of *C. graminicola* on barley leaves produced in the temperature range of 15°C to 35°C, but most suitable temperature is 25-30°C (Skoropad, 1967). Lapp and Skoropad (1978) concluded that location of appressoria on barley leaf is dependent on the physical surface structure of the leaf and is not dependent on any chemical differences in the host

cuticle. Dey (1919) observed that agar surfaces do not always provide sufficient stimulus for differentiation of appressoria in *Colletotrichum*. It was confirmed by Staples *et al.* (1976) and Van Dyki and Mims (1991) working on *C. truncatum* that thigmotrophic stimulus is responsible for differentiation of appressoria. Although the role of contact stimulus also was reported in *C. lindemuthianum* (Dey, 1919) but according to Leach (1923) it could be blocked by the addition of nutrient.

Chowdhury (1936) described the size of appressoria ranging from 8-15 μm in diameter whereas Mordue (1967) described them as irregularly obovate to clavate or ellipsoid, ochraceous tawny to cinnamon brown measuring 6-33 (mean 15.64) μm x 4-25 (mean 11.2) μm wide with single germ pore.

2.2.3 Setae

The production of setae during the formation of the acervulus in the genus *Colletotrichum* has for sometime been regarded as of doubtful value in distinguishing the genera *Colletotrichum* and *Gloeosporium* (Alexopoulos and Mims, 1979).

Setae appear in mature acervulus. They are mixed with the conidiophores in the acervulus and are long, dark brown to black, thick walled processes or bristles, somewhat pale at the tip. They are unbranched 2 to 5 septate and measure from 4-6 μm in width and upto 170 μm in length. Usually setae are broader at the base, often bulbous

and gradually tapering toward the apex (Chowdhury, 1936). Similar findings were made by Mordue (1967).

According to Von Arx (1957) *Colletotrichum* may or may not produce setae. The absence of setae in conidiomata in some *Colletotrichum* species to some extent can be controlled genetically as in *C. musae* and *C. gossypii* Southw. var. *cephalosporioides* A.S. Costa which consistently failed to produce setae (Sutton and Waterson, 1970; Sutton, 1992). In addition environmental condition, specifically atmospheric humidity play a role in setae production. Frost (1964) reported that at near saturated humidity *Colletotrichum linicola* produced acervuli on host plant without setae, but at lower humidity of approximately 95 per cent relative humidity acervuli with several setae were formed. Maiello (1988) reported that development of setae is related to the type of acervulus primordium initiated in that particular *Colletotrichum* species.

Southworth (1891) observed the truncate apex of setae which produced conidia. Lenne *et al.* (1984) also reported two types of setae, those which are characterized by truncate, near hyaline apices and produced conidia are called fertile, whereas which are darker usually pointed apices and did not produce conidia called sterile setae. They conducted their work with 93 isolates of 10 different species including *C. graminicola.*

2.3 CULTURAL VARIABILITY

Colonies on potato dextrose agar are grey, even, compact, felty to wooly, occasionally tufted or sparse but showing no zonation. Reverse of

the colony lilac to grey or greenish-grey, darker with age because of the formation of appressoria. Spore masses colourless, grey or dirty pink to salmon orange (Mordue, 1967). Chowdhury (1936) worked with various nutrient media and found that maize meal, oat meal, Dox's and Hopkins agar favoured aerial growth with copious sporulation. According to Chohan (1967) colonies were appressed and fluffy and the amount of mycelium ranged from fair to very good and greyish white.

Saifulla and Ranganathaiah (1990) observed that maximum radial growth of the anthracnose pathogen from sorghum was in host seed extract followed by oat meal agar. Similarly Jamil and Nicholson (1989) tested radial growth rate of different isolates of *C. graminicola* from shattercane, sorghum and corn on seven different media and found that the isolates grew best on malt agar.

Best colony diameter was found in 3 sorghum isolates of *C. graminicola* at minimum 8 hr light and 16 hr dark treatment (Mishra and Siradhana, 1980). They also found that the significant differences in colony diameter of 3 isolates in all light exposure.

Sporulation was good when cultures of *C. graminicola* were kept in alternate light and dark cycle at 100 per cent relative humidity (Chowdhury, 1936) as well as temperature of 30°C to 35°C (Saifulla and Ranganathaiah, 1990). Ghouse (1975) observed profuse mycelial growth at the temperature of 25-30°C and maximum sporulation at 30°C.

Studies of LeBeau *et al.* (1951) indicated that *C. graminicola* isolates showed more luxuriant growth and sporulation on PDA but did not mention any specific light or temperature conditions. But according to Lilly and Barnet (1951) a fungus produces reproductive bodies and spores under nutritional condition which are often quite different from those which are optimum for vegetative growth. Mishra and Siradhana (1980) observed that light exposure favoured growth and sporulation of the pathogen.

2.4 PATHOGENIC VARIABILITY

Colletotrichum graminicola is a highly variable pathogen (Ali and Warren, 1992). Although LeBeau and Coleman (1950) studied the variation in aggressiveness among different isolates of *C. graminicola* from sorghum, Harris and Johnson (1967) were the first to suggest the possible existence of physiological races of *C. graminicola* in USA. In addition some sorghum cultivars were reported to react differently in Texas, Mississippi and Georgia (Frederiksen and Rosenow, 1971). Results of the 1976 International Sorghum Anthracnose Virulence Nursery (ISAVN) suggested the presence of different races of *C. graminicola* in the United States and Nigeria (King and Frederiksen, 1976). Similarly all 13 entries of the 1981 ISAVN were susceptible to anthracnose at Pantnagar, North India, suggesting that the Indian population of the pathogen was different from that in the United States (Frederiksen, 1984).

Nakamura (1982) gave the first positive evidence for the existence of physiological races in Brazil and identified five races of *C. graminicola*.

Later many more races have been identified from Brazil (Ferreira *et al.*, 1986; Casela *et al*, 1992; 1993 and 1995).

Since 1980, 12 races have been reported from Brazil (Nakamura, 1982; Ferreira and Casela, 1986), 10 from Northern Nigeria (Ozolua *et al.*, 1986), 11 from USA and Puerto-Rico (Ali and Warren, 1987; Cardwell *et al.*, 1989), 9 from India (Pande *et al.*, 1991) and two from Western Africa (Thomas, 1991). Thus in 10 different studies from 1967 to 1991, 44 races/pathotypes have been reported on 43 sorghum genotypes from different parts of world.

In India, Pande *et al.* (1991) first reported the presence of different races of the pathogen in different states. Later variability in pathogenicity was observed even among different isolates within a state (Rao *et al.*, 1998). Similar observations were made by Mathur *et al.* (1998) with 26 *C.graminicola* populations collected in from Andhra Pradesh from local land races as well as hybrids and differentiated them into seven pathotypes. In another investigation the same workers found significant pathogenic heterogeneity in the single-lesion population of *C. sublineolum* (= *C. graminicola*) from a single sorghum cultivar CSH-9 collected from different parts of Maharashtra (Mathur *et al*, 1998).

Mathur *et al.* (1997) further demonstrated intra- population variability among 12 monoconidial cultures derived from a single isolate in their morphological, cultural and pathogenic characteristics.

2.4.1 Disease rating scale

Sorghum anthracnose is evaluated with separate qualitative and quantitative scales. For qualitative rating Cardwell *et al.* (1989) classified scores into three groups : 1-2.5 = resistant (R); 2.6-3 = moderately susceptible (MS) and 3.1-5 = susceptible (S). Pande *et al.* (1991) classified as 0 = resistant (R), 1-3 = hypersensitive resistant (HR), and 4-6 = susceptible (S).

For quantitative rating Harris and Sowell (1970) used a 0-5 scale with an interval of 0.5, where 0 = no lesion, and 5 = 91-100% leaf area covered with lesions. Ferreira and Warren (1982) used a 1-5 scale where 1 = no lesion and 5 = 100% leaf area covered with lesions or plant dead. Cardwell *et al.* (1989) used 1-5 scale where 1 = unblemished leaf, >1-3 = hypersensitive lesions without acervuli, and 3-5 = lesions with acervuli covering 100% leaf area. Pande *et al.* (1991) used a 0-6 scale, where 0 = no lesion, and 6 = coalescing necrotic lesions with abundant acervuli.

A new rating scale was proposed in 1992 through International Sorghum Anthracnose Virulence Nursery (ISAVN); namely 1-9 scale for severity (where 1 = no lesions and 9 = >75% leaf area covered with susceptible lesions); and a R, MR and S scores for reaction types (where R = presence of fleckings, but no lesions; MR = hypersensitive lesions without sporulation and S = lesions with acervuli) (Thakur, 1995). This scale has now been accepted and being used internationally (Mathur *et al.*, 1997; Rao *et al.*, 1998).

2.5 MOLECULAR VARIABILITY

For detection of molecular variability Random Amplified Polymorphic DNA (RAPD) analysis is a modern, fast and popular technique. It uses the polymerase chain reaction (PCR) with short nonspecific primers under condition of modest stringency. Single primer of arbitrary nucleotide sequence is used to uncover molecular variability and requires no prior sequence information (Williams *et al.*, 1990). The net effect of this technique is to amplify several regions of the genome each of which are flanked by the specified priming sites. Most variation is assumed to be derived from single base pair mutations in the priming site. The basic assumption is that comigrating fragments between accessions represent homologous regions of the genome (William *et al.*, 1996). In a comparative study between RFLP and RAPD markers with *Brassica napus* it was concluded that RAPD markers have the same resolving power as RFLP markers (Hallden *et al.*, 1994).

At present RAPD analysis has proven as useful technique for detection of polymorphism between races of plant pathogenic fungi (Jones and Dunkle, 1993; Grajal-Martin *et al.*, 1993; Ouellet and Seifert, 1993; Manulis *et al.*, 1994).

Guthrie *et al.* (1992) used this technique to differentiate the isolates of *C.graminicola* from sorghum and Johnson grass and concluded about the potential importance of geographical origin. Greater genetic diversity was found using RAPD analysis than virulence analysis on host differentials (Casela *et al*, 1993).

DuTeau and Leslie (1992) used arbitrary sequenced primers to differentiate isolates belonging to the six mating populations within *Fusarium* section Liseola (*Gibberella fujikuroi*). Isolates of other *Fusarium* species, like *Fusarium graminearum*, *F. oxysporum* f.sp. *dianthi* and *F. oxysporum* f.sp. *pisi* have been successfully differentiated by RAPD analysis (Ouellet and Seifert, 1993; Manulis *et al.*, 1994; Grajat-Martin *et al*, 1993).

Fischer *et al.* (1995) reported that isolates of *Ascochyta rabiei* could be identified using RAPD. Similar conclusions were also drawn by other workers (Udupa *et al.*, 1997).

Isolates of *Cochliobolus carbonum* from maize were distinguished by PCR amplification with arbitrary and gene-specific primers (Jones and Dunkle, 1993). Abadi and coworkers (1996) studied 13 isolates of *Exserohilum turcicum* from maize and 5 from Johnson grass by using RAPD markers and they correlated their work with virulence and aggressiveness on both maize and johnson grass.

During their work with witches broom pathogen *Crinipellis perniciosa* from several hosts Andebrhan and Furtek (1994) detected the differences among monospore culture from same basidiocarp by RAPD analysis.

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MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

Experiments described in this chapter were conducted at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru, Andhra Pradesh, 502 324, India.

The materials and methods used in the present investigations were broadly described under the following heads.

1. Sterilization of materials and equipments
2. Collection of diseased samples
3. Isolation and pure culture of the pathogen
4. Morphological studies
5. Cultural studies
6. Studies on pathogenicity
7. Molecular analysis
8. Statistical analysis

3.1 STERILIZATION OF MATERIALS AND EQUIPMENTS

Liquid materials,such as media and distilled water were sterilized in an autoclave at 121°C and 15 pound per square inch (p.s.i.) for 20 min. Glass wares and other equipments were sterilized in a oven at 170°C for 1 hr after wrapping in aluminium foil.

For surface sterilization 0.1% sodium hypochlorite (NaOCl) was used for plant materials such as leaf, seed etc., and rectified spirit used for

other equipments like inoculation-needles, forceps, inoculation chamber, hands etc.

Soil (Black soil : Sand : FYM at 3:2:2 by Vol.) was sterilized using autoclave at 121°C and 15 p.s.i. for 20 min for two cycles at 24 hr. interval. Soil with sufficient moisture but in friable condition was kept in 20 kg gunny bag and stitched properly before loading in autoclave.

3.2 COLLECTION OF DISEASED SAMPLES

Diseased samples of sorghum anthracnose collected from 4 states (Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu) of India, preserved at ICRISAT, Patancheru at 4°C in refrigerator and used for studies for their variability (Table 1.).

3.3 ISOLATION AND PURE CULTURE

3.3.1 Isolation

Leaves with lesions of anthracnose (caused by *Colletotrichum graminicola*) were selected for isolation of the pathogen. One grain sample was also used for isolation. The samples were washed with distilled water. Small bits of diseased leaf spots adjoined with some healthy tissue were cut with the help of scissor and surface sterilized with 0.1% sodium hypochlorite for 2 min. The leaf bits were thoroughly washed with sterile distilled water and dried in an aseptic condition. The leaf bits were transferred aseptically onto previously sterilized petriplates containing oat meal agar (OMA) medium and incubated at 25°C. For grain isolate, diseased

grains were soaked in sterile distilled water for 30 min at aseptic condition and then surface sterilized as leaf bits and transferred on the media plates.

3.3.2 Pure culture

The cultures were purified by subculturing the organism from the centre of colony touching the inoculation needle tip at orange coloured acervuli. As different monoconidial cultures derived from a leaf isolate showed variability in morphology and pathogenicity among them as well as from parent culture (Mathur *et al.*, 1997), bulk cultures were used for all leaf isolates in this study and only for two isolates (grain and midrib) monoconidial cultures have been used for studies along with respective parent isolates. For single spore isolation, a diluted spore suspension (approximately 3000 spores/ml) prepared in sterile distilled water was poured on solidified yeast extract agar plate. Previously yeast extract agar media was poured in sterilized petri plates as a thin layer. Since both water agar and conidia are hyaline so yeast extract agar was used instead of water agar for single spore isolation because it is yellow in colour. One ml of spore suspension was poured in each media plate using sterilized pipette and the plate tilted slowly for uniform spread of the suspension and kept in laminar flow for 15 min. The extra water was poured off slowly from the plates and those plates incubated at 25°C for 16 hr under fluorescent light.

The plates were observed under microscope for single germinated spores using 10x objective. This objective was replaced by sterilized 'dummy' objective and brought down on media plate to get a circular

marking around the germinated spore. The marked spores were then transferred to OMA plates and incubated at 25°C for 7 days under constant fluorescent light.

3.3.3

Table 1: Details of *Colletotrichum graminicola* isolates collected from 4 states of India

S.No.	Isolate designation	Location/state
1.	Cg 20	Koppal /Karnataka
2.	Cg 75	Peddapur /Andhra Pradesh
3.	Cg 94	Thalval /Maharashtra
4.	Cg 118	Mariwad /Karnataka
5.	Cg 130	Jedcherla /A.P.
6.	Cg 138	Patancheru /A.P.
7.	Cg 150	Pudur /Tamil Nadu
8.	Cg 158	Thamaraikulum /Tamil Nadu
9.	Cg 226 (grain)	Patancheru /A.P.
10.	Cg 226-3 (monoconidial isolate of Cg 226)	Patancheru /A.P.
11.	Cg 227	Patancheru /A.P.
12.	Cg 227-3 (monoconidial isolate of Cg 227)	Patancheru /A.P.

Isolates were selected on the basis of geographical distribution, plant part (lamina, midrib, grain) affected and the types of symptoms they produced. Isolate Cg 118 produced large lesions (Plate 1) with dark red to black margin surrounding a white centre consisting of numerous black dots (acervulus) whereas Cg 227 produced symptom only on midrib (Plate 2). Isolate Cg 150 from Pudur/ Tamil Nadu, showed two types of

lesions on leaf lamina - large and small (Plate 3). From large lesions Mathur *et al.* (1997) isolated the pathogen and identified as *Colletotrichum gloeosporioides*. So in this study smaller lesion was used which is caused by *Colletotrichum graminicola*. Cg 226 is the only grain isolate used (Plate 4). For other isolates symptoms were more or less similar as described in symptoms of the disease.

Cg 226-3 and Cg 227-3 were the monoconidial isolates of Cg 226 and Cg 227, respectively. Ten single germinated conidia were isolated in 10 OMA plates for each isolate and randomly marked as 1 to 10. Incidentally, plate number 3 of both isolates gave colonies of medium diameter in comparison to others with profuse sporulation and therefore were selected for further work.

3.3.4 Composition and preparation of oat meal agar (OMA) medium

Commercial oat meal agar (Himedia)	-	38 g (18 g oat meal+ 20 g agar)
Agar (Himedia)	-	5 g
Distilled water	-	1 L

Thirty eight grams of oat meal agar and 5 g of agar were mixed in 1 L of distilled water and boiled for 8 min at high power in Nobel auto oven. While boiling it was stirred twice to prevent settling of medium at the bottom of beaker. After boiling the medium was filtered through muslin cloth and poured 250 ml medium in conical flasks (500 ml) and sterilized in an autoclave 121°C for 20 min at 15 p.s.i.

Oat meal broth medium

Champion white oats (Hindustan Vegetable Oil Corporation Limited)	-	10 g
Distilled water	-	1 L

Yeast extract agar medium

Yeast extract agar (Himedia)	-	50 g (contain yeast extract 10 g; peptone 20 g and glucose 20 g
Agar (Himedia)	-	15 g
Distilled water	-	1 L

Both media were sterilized after preparation by autoclaving.

3.4 MORPHOLOGICAL STUDIES

3.4.1 Preparation of lactophenol

Phenol (crystals)	-	20 g
Lactic acid	-	20 ml
Glycerol	-	40 ml
Distilled water	-	20 ml

Cotton blue crystals (0.05 g) was added in 50 ml lactophenol and mixed by vortex mixer to dissolve it. The stain was then filtered through Whatman No.1 filter paper to make it dust free and transferred to a stoppered bottle.

3.4.2 Preparation of slides

A small amount of pure culture was taken using a sterile needle and transferred to a clean slide. The culture was taken from four positions of the culture plate, two at right angle to each other, one from very close to the inoculation point and another from the midpoint of the radius. Total 3 culture plates of each isolate were used for the morphological studies after 10 days of incubation at 25°C. The culture was stained with 0.1% lactophenol-cotton blue and observed for the acervuli, setae, appressoria, sclerotia and conidia using a compound microscope (Olympus CH-2, Japan).

3.4.2.1 Acervulus

Formation of acervulus and the pattern of conidiogenous cells were observed. The following observations were made 10 days after incubation in all the isolates designated as.

i)	Acervulus - light brown to dark brown conidiogenous cell - clearly distinguished producing conidia		Distinct
ii)	Acervulus conidiogenous cell	- light yellowish, indistinguishable	Indistinct

3.4.2.2 Setae

Presence of setae in the acervulus and their number per acervulus in 10 randomly selected acervulus were observed 10 days after culturing at 25°C. Length and breadth of 50 random setae were measured using ocular micrometer.

3.4.2.3 Appressoria

Presence or absence of appressoria, shape and size of appressoria were observed using 1000X magnification in 10 day-old culture. For measurement of size, 10 randomly selected appressoria were recorded using ocular micrometer.

3.4.2.4 Sclerotia

Presence or absence of sclerotia were observed in 10 days old culture and if absent, the cultures were once again examined 20 days after incubation for further confirmation. Compact, black and round sclerotia were considered as mature sclerotia whereas sclerotia with loosely woven brown coloured mycelia were considered as young, forming or immature sclerotia.

3.4.2.5 Conidia

The shape and size of conidia were recorded in 50 randomly selected conidia using 1000X magnification. Shape of conidia was recorded whether conidia were typical falcate or fusoid or straight produced by different isolates.

3.5 CULTURAL STUDIES

All the 12 isolates of *C. graminicola* were grown on OMA medium for the cultural studies.

3.5.1 Growth pattern and colony colour

Characteristics of growth and colour of colony were studied with the following observations:

(i) Colony produced aerial fluffy, raised mycelium or mycelium submerged in growth.

(ii) Mycelium nodular, wooly and undulated in appearance or dendroid, compact and felty.

(iii) Margin of the colony sharply delimited, distinct or irregular diffused.

(iv) The colour of the colony whether grey, white, or salmon orange.

(v) Conidia formed in orange masses or greyish masses.

3.5.2 Colony growth

Twenty ml of the OMA medium was poured in sterilized petriplates and allowed to solidify. Mycelial discs of 5 mm diameter were cut from the margin of the 7-day-old culture of *C. graminicola* for each isolate and placed at the centre of the petriplate under aseptic conditions.

The plates were incubated for 10 days at 25°C and the diameter of the colony was measured using a ruler. Three replications of each isolate were maintained for study.

3.5.3 Sporulation

Five mm^2 disc of the fungal culture were cut by a sterile cork borer from the centre to the margin at three different equidistant places

along the radius. The three discs were taken in a test tube containing 10 ml distilled water and crushed by a glass rod. The suspension was shaken by a vortex mixer to dislodge the conidia. Spore suspension (0.02 ml) was placed with a graduated 0.1 ml pipette in one chamber of the haemocytometer. The number of the spores in a small square at the centre were counted and calculated for the total number of spores (=nx10^4; where n=no. of spores per small square) in 1 ml of the spore suspension. Two replications of each isolate were observed.

3.6 PATHOGENICITY STUDIES

Studies on pathogenicity were carried out at glass house conditions at ICRISAT, Patancheru. Plastic pots of 7" x 7" size were washed thoroughly with tap water. A square shaped paper was placed at the bottom of each pot and filled up with around 3 kg of sterilized black soil : sand : Farm Yard Manure (FYM) mix (3:2:2 by volume). Ten grams of diammonium phosphate was added to the top soil and mixed in each pot.

3.6.1 Host differentials

Six sorghum genotypes were selected from those lines used as host differentials in the International Sorghum Anthracnose Virulence Nursery (ISAVN) (Thakur, 1995).

1. A 2267-2
2. IRAT 204

Resistant

3. IS 8354
4. IS 3758

Moderately resistant

5. IS 3089
6. IS 18442

Suscpetible

Seeds were surface sterilized with 0.1% mercuric chloride for 4-5 min, washed thoroughly with distilled water and dried at room temperature before sowing. Seeds were sown equidistantly at 5 places in each pot and watered regularly with soft water. After one week plants were thinned keeping 5 healthy plants per pot and a week later Hoagland nutrient solution (Hoagland *et al*, 1950) was supplied (100 ml/pot) for good growth of the plants. Two pots for a single sorghum line were used to inoculate with an isolate.

3.6.2 Preparation of inoculum

The inoculum of each isolate was prepared by flooding the 12 days old culture plates of *C. graminicola* grown on OMA medium with distilled water and scrapping superficially with a scalpel. The spore suspension was filtered through muslin cloth. The conidial concentration was established using haemocytometer. A concentration of $1x10^5$ conidia/ml was used for inoculation. Two hundred fifty ml of spore suspension was prepared for each isolate. Tween 20 (Polyoxyethylene sorbitan monolaurate), a surfectant and dispersing agent was added to the spore suspension @ 0.1% to enable uniform spread of inoculum on the leaves.

3.6.3 Inoculation

Twenty one days old plants (5-6 leaf stage) were inoculated by each isolate as a separate batch consisting of two pots of each host

differential. The spore suspension was sprayed using a hand atomiser. During inoculation,polythene screen was used to prevent cross inoculation. After air drying, plants were kept in the humid chamber (RH>90%) at 25 $\pm$ 2°C for 24 hrs. Later these pots were shifted to glasshouse bench arranging one isolate as a batch and a high relative humidity (>90%) was maintained 8 hrs per day. Old and dried leaves were removed leaving 3-4 leaves for observations. Five plants in each pot were tagged with polythene tape of different colours for recording latent period, disease reaction and disease severity from same plant at different intervals (Mathur *et al.*, 1997).

3.6.3.1 Latent period

Latent period was taken as time in days from inoculation to appearance of first chlorotic/necrotic lesion for each isolate-line combination. The top healthy leaves were examined for the appearance of chlorotic or necrotic lesion every day upto 7 days after inoculation.

3.6.3.2 Disease reaction

Evaluation of disease was done 14 days after inoculation. Disease reactions were scored and the virulence of pathogen was corresponded with disease reaction and expressed as numerical value (Mathur *et al.*, 1997).

Symptoms	Disease Reaction	Numerical score
No symptoms or chlorotic flecking	Resistant (R)	1
Hypersensitive lesions red spots or necrotic spots without acervuli (Plate 5)	Moderately resistant (MR)	2
Necrotic lesion with sporulation (Plate 6)	Susceptible (S)	3

3.6.3.3 Disease severity/Aggressiveness

A 1-9 scale (Mathur *et al.*, 1997) was followed (Plate 7).

Scale	Disease severity
1	No lesion or presence of chlorotic flecks
2	1-5% leaf area covered with lesions
3	6-10% leaf area covered with lesions
4	11-20% leaf area covered with lesions
5	21-30% leaf area covered with lesions
6	31-40% leaf area covered with lesions
7	41-50% leaf area covered with lesions
8	51-75% leaf area covered with lesions
9	> 75% leaf area covered with lesions

3.6.3.4 Virulence index

The numerical values of disease severity, disease reaction and latent period were used to calculate the virulence index with the following formula (Mathur *et al*, 1997).

Virulence Index (VI) = (Virulence x Aggressiveness) x Latent period^{-1}

3.7 MOLECULAR ANALYSIS

Random Amplified Polymorphic DNA (RAPD) analysis was used to detect the variability among the isolates of *C. graminicola*. Standard protocols were used for the isolation of DNA and gel electrophoresis (Williams *et al*, 1990).

3.7.1 Multiplication of cultures

The isolates of *C. graminicola* were multiplied on oat meal broth medium (0.1%). Each isolate was inoculated in 6 conical flasks (250 ml) containing 125 ml medium. Inoculum was collected from the margin of the colony by scalpel and inoculated to flasks directly. The flasks were incubated at 25°C at 125 rpm for 7 days in an illuminated, refrigerated incubator shaker (New Brunswick Scientific Co.).

3.7.2 Composition of chemicals used for DNA extraction

3.7.2.1 Tris-HCl 0.5M, pH 8.0:

60.55 g of Trizma base (Tris [Hydroxymethyl] amino methane MW=121.1) was dissolved in 500 ml of distilled water. The pH was adjusted

with concentrated HCl and the volume was made upto 1000 ml and autoclaved.

3.7.2.2 EDTA 0.5M, pH 8.0

186 g of Ethylene diamine tetraacetic acid (EDTA; MW = 372.2) was dissolved in 500 ml of distilled water. The volume was made upto 1000 ml with distilled water and autoclaved. The pH was adjusted using sodium hydroxide.

3.7.2.3 CTAB buffer

Tris HCl 0.5 M, pH 8.0	-	400 ml
EDTA 0.5 M, pH 8.0	-	40 ml
CTAB (Hexadecyl trimethyl-ammonium bromide)	-	20 g
NaCl	-	82.16 g

Sterile distilled water was added to make the volume upto 1000 ml.

3.7.2.4 T_{80} E_{10} buffer

Tris HCl 0.5 M, pH 8.0	-	10 ml
EDTA 0.5 M, pH 8.0	-	2 ml

Distilled water was added to make the volume upto 100 ml and autoclaved.

3.7.2.5 T_{10} E_1 buffer

Tris HCl 0.5 M, pH 8.0	-	20 ml
EDTA 0.5 M, pH 8.0	-	2 ml
Distilled water	-	To make the volume 1000 ml and autoclaved.

3.7.2.6 TAE (Trizma acetic acid EDTA) buffer, pH 7.8 (10X):

Tris base	-	48.44 g
Sodium acetate	-	16.4 g
EDTA 0.5 M, pH 8.0	-	36 ml

pH was adjusted to 7.8 with acetic acid and the volume made up to 1 lt and autoclaved.

3.7.2.7 T_{50} E_{10} buffer + RNase

T_{50} E_{10} buffer	-	100 ml
RNase	-	1 ml (5 mg of RNase dissolved in 1 ml of sterile distilled water)

3.7.3 DNA Extraction

The mycelium of each isolate was harvested by filtering through Mira cloth (Calbiochem, USA) and washed thoroughly with distilled water. Excess water was removed using blotting paper. Mycelial mass was made into small pieces and ground under liquid nitrogen with a prechilled pestle and mortar. Powdered sample was transferred to 50 ml screw cap tube

containing 15 ml CTAB buffer and shifted in a water bath at 65°C for 30 min.

After cooling the tubes to room temperature, 15 ml of chloroform and isoamyl alcohol (chloroform : isoamylalcohol = 24:1) was added to each tube and mixed thoroughly. The content was transferred into Sorvall tubes and kept for centrifugation at 6000 rpm for 15 min in Sorvall RC 5C superspeed refrigerated centrifuger.

The supernatent was collected carefully in another tube and 15 ml of chloroform:isoamylalcohol (24:1) was added and the procedure repeated.

Finally the aqueous layer was collected in screw cap tube and 15 ml of isopropanol was added to it. The content was mixed by tilting the tubes slowly and kept at -20°C for 20 min to allow the DNA to settle down.

The DNA in the liquid was spooled out with the help of a bent Pasteur pipette rod and transferred to Falcon tubes (13 ml) containing 5 ml of 70% ethanol and centrifuged at 3000 rpm for 5 min. The alcohol was discarded and again 5 ml of 70% ethanol was added to wash the DNA and the above procedure was repeated.

The pellet at the bottom of tubes was dried briefly in DNA vac (Sarvant DNA speed vac Model 110) at medium speed for 3 min.

Two ml of T_{50} E_{10} buffer + RNase was added to the pellet and the tubes were kept at 37°C for 1 hr. and left over night at 4°C.

One ml of chloroform : isoamylalcohol (24:1) and 1 ml of phenol were added to these tubes, mixed thoroughly and centrifuged at 3000 rpm for 5 min. The aqueous layer was collected and the procedure repeated.

Again 2 ml of chloroform : isoamylalcohol (24:1) was added to the aqueous layer and centrifuged at 3000 rpm for 5 min.

The aqueous layer was collected in Falcon tubes and 100 µl of 3 M sodium acetate and 3 ml of isopropanol was added to each tube, mixed thoroughly and kept at -20°C for 20 min.

DNA was spooled out and transferred to an 1.5 ml eppedorf tube containing 1 ml of 70% ethanol. The tubes were centrifuged at 10000 rpm for 10 min, ethanol was decanted carefully. Again 1 ml of ethanol was added to the pellet and centrifuged and same procedure was repeated.

The pellet was dried in DNA vac at medium speed for 2 min. Depending upon the quantity of DNA 50 µl to 250 µl $T_{10}E_1$ buffer was added to dissolve the DNA.

3.7.4 Measurement of DNA concentration

The concentration of DNA was measured spectrophotometrically. Two ml of $T_{10}E_1$ buffer was taken in the cuvette and 5 µl of DNA sample was added and read at 260 nm and 280 nm. The concentration was calculated using the formula

$$\text{Concentration of DNA} = \frac{\text{OD} \times 50}{5} \mu g/ul \text{ (OD at 260 nm)}$$

$$= \frac{\text{OD} \times 50}{5} \times 1000 \text{ ng/ul}$$

The samples were diluted to make 12.5 µg/ul by adding $T_{10}E_1$ buffer.

Table 2: Final DNA product and its dilution

Isolate designation	Wt.of wet mycelium (gm)	Vol. of DNA suspension (µm)	DNA concentration (ng/ul)	Dilution factor to get DNA of 12.5 (µg/µl)
Cg 20	1.43	100	880	1:70
Cg 75	4.74	100	160	1:13
Cg 94	3.21	50	420	1:35
Cg 118	4.51	250	830	1:65
Cg 130	2.6	100	970	1:80
Cg 138	6.65	100	1600	1:130
Cg 150	2.8	200	1330	1:110
Cg 158	4.3	150	920	1:75
Cg 226	4.47	100	450	1:37
Cg 226-3	5.46	250	1510	1:125
Cg 227	2.4	100	460	1:38
Cg 227-3	7.89	200	1660	1:135

3.7.5 Purity of isolated DNA

The purity of the DNA was checked by running on an agarose gel. The following steps were followed.

3.7.5.1 Agarose gel (1.4%)

Agarose	-	0.98 g	0.64g
TAE buffer (0.5 X)	-	70 ml	80ml
Ethidium bromide	-	10 µl	4µl

1X

Agarose (0.98g) was mixed in 70 ml of TAE buffer (0.5X) and heated in a microwave oven to dissolve the agarose in TAE buffer. 10 µl of Ethidium bromide was added after cooling.

3.7.5.2 Casting of agarose gel

Molten agarose was poured in the gel casting tray (14 cm x 10 cm) carefully avoiding formation of air bubble. A clean Teflon comb with 13 teeth was placed prior to pouring of agarose suspension. After solidification the gel was kept in the electrophoresis tank under TAE (0.5X) buffer and the comb was removed.

3.7.5.3 Loading of samples

Three microlitres DNA and 2 µl of loading buffer was mixed in a 0.5 ml eppendorf. These prepared samples were loaded in the wells slowly with pipette and the assembly was connected to the Biorad power supply

unit. The gel was run at 70V for 45 min. After completion of run the gel was checked on transilluminator for the purity and concentration of the DNA samples.

Five samples Cg 75, Cg 118, Cg 138, Cg 158 and Cg 226-3 produced a smear on gel indicating the impurity of the samples. Hence they were further purified. The ratio between ODs at 260 nm and 280 nm also gave an idea about the purity of DNA. When the OD ratio (260 nm/280 nm) was above 1 and approached to 2 it indicated as good quality DNA.

3.7.5.4 Purification of the samples

To DNA samples 0.5 ml of T_{50} E_{10} + RNase was added and kept at 37°C water bath for 1 hr. to dissolve the DNA properly. Equal volume of phenol and chloroform (0.25 ml each) was added to the samples, mixed and centrifuged as before. The aqueous layer separated and the procedure was repeated once and 0.5 ml of chloroform : amylalcohol (24:1) added to the aqueous layer and centrifuged again.

Fifty microlitres of 3 M sodium acetate and 1 ml of isopropanol was added to the aqueous layer and kept at -20°C for 20 min. DNA was spooled and transferred to 1 ml of 70% ehthanol in eppendorf tube for washing. Alcohol was decanted after centrifugation. The procedure repeated once.

Pellet was dried and DNA was dissolved in $T_{10}E_1$ buffer.

3.7.6 Amplification of DNA using Polymerase Chain Reaction (PCR)

The reaction composition for the PCR was prepared by mixing the following reagents for each DNA sample.

10X buffer (Promega Corp., USA)	-	2.5 µl
25 mM $MgCl_2$	-	1.0 µl
0.2 mM dNTPs (New England Biolab, USA)	-	2.5 µl
0.5 µM primer (Operon Technology Incorporated, USA)	-	2.5 µl
1 Unit of Taq polymerase (Promega Corp., USA)	-	0.5 µl
Template	-	25 ng (2 µl of diluted sample)
Sterile distilled water (To make the total volume 25 µl)	-	14 µl

Master mix was prepared by mixing all components excluding template DNA for all 12 isolates and 23 µl taken into each PCR tube. Template DNA (2 µl) was added in each tube and capped. The tubes were placed in a Thermocycler (Perkin-Elmer, USA).

The amplification reaction was carried out using the DNA Thermal Cycler with the following temperature profile: cycle started after the temperature reached to 100°C then 45 amplification cycles were conducted with 1 min at 92°C (for denaturation), 1 min at 35°C (for annealation) and 2 min at 72°C (for extension).

3.7.6.1 Primer

A 10-base primer OPA8 with base sequence of 5'GTGACGTAGG3' was used in this RAPD analysis.

3.7.7 Electrophoresis

3.7.7.1 Preparation of sample for electrophoresis

The samples for electrophoresis were prepared by mixing 5 µl of loading buffer in each amplified product. Bromophenol blue indicates the position of the run and sucrose present in the buffer helps the sample to settle down at the bottom of the well.

3.7.7.2 Gel electrophoresis

Agarose gel (1.4%) with 13 wells and 14 cm x 10 cm size was prepared for electrophoresis and placed in electrophoresis tank (Bio-Rad, USA) pouring 0.5 X TAE buffer sufficient to immerse the gel completely. Eighteen microlitres of sample was loaded in a well using a micropipette. In the last well 7 µl of a 100 base pair marker was loaded. This gel was electrophoresed at 50V for 2 1/2 hrs.

The gel was checked after run on transilluminator. Excess ethidium bromide was washed by distilled water. Gel was placed in distilled water in a tray and then washed keeping on a shaker with slow rotation for 15 min. After washing the gel was kept on the transilluminator (PhotoDyne Incorporated, USA) and photographed using Polaroid camera.

The RAPD procedure was repeated thrice following similar protocol to test the reproducibility of the results.

3.8 STATISTICAL ANALYSIS

Range, mean, standard deviation, coefficient of variation and standard error of means were determined for all measurements of morphological and cultural studies to compare significant differences among isolates. Least significant differences (LSD) or critical differences were calculated to compare significant differences among isolates and sorghum lines for latent period, aggressiveness and virulence index. Analysis of variance was done to determine the significant interactions of isolate and host differentials in two experimental runs separately. The error mean square of two experimental runs was subjected to F-test. The error variances were considered homogenous as the highest error mean square was not three-fold larger than the smallest error mean square (Gomez and Gomez, 1984). The data from the two experimental runs were pooled and means were estimated through analysis of variance. All these analysis were done using computer package programme SAS (1985).

Dendrograms were prepared by cluster analysis for molecular variability and virulence index using GENSTAT (1986).

Plate 1: Big size lesions of Cg 118 isolate of *C. graminicola* on leaf lamina collected from Mariwad, Karnataka

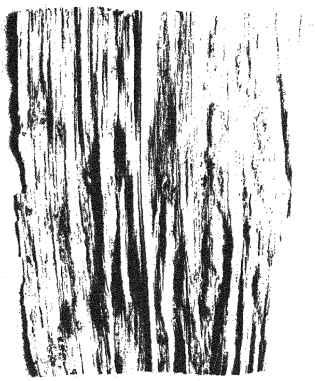


Plate 1

Plate 2: Lesions of Cg 227 isolate of *C. graminicola* on midrib, collected from Patancheru, Andhra Pradesh

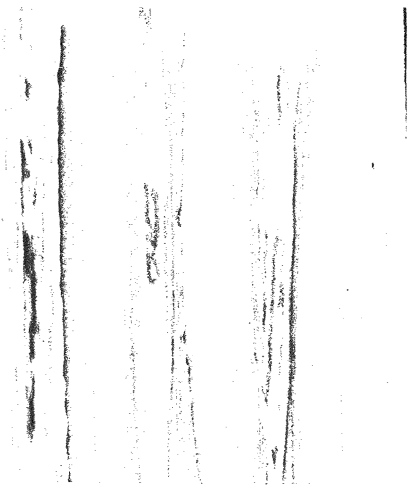


Plate 2

Plate 3: Big (arrowheads) and small (arrows) lesions of Cg 150 isolate of *C. graminicola* on leaf lamina collected from Pudur, Tamil Nadu

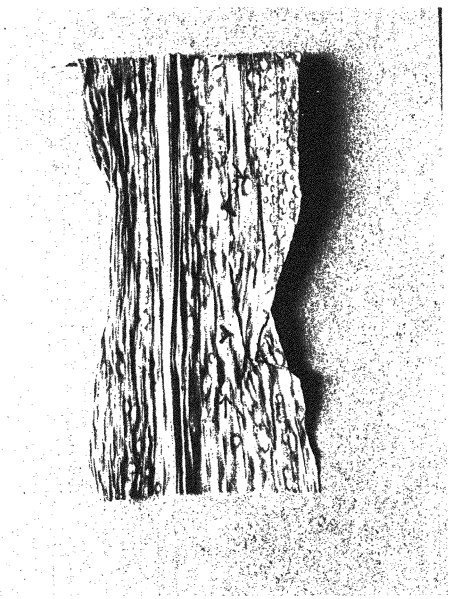


Plate 3

Plate 4: Black lesions of Cg 226 isolate of *C. graminicola* on grains at maturity collected from Patancheru, Andhra Pradesh

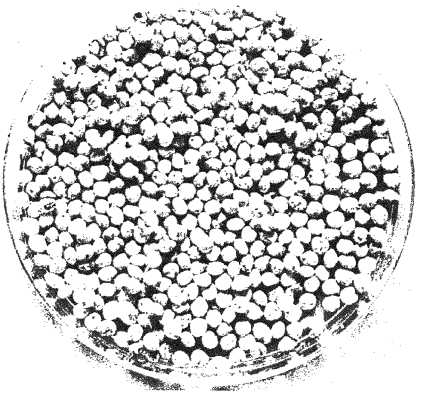


Plate 4

Plate 5: Symptom of moderately resistant reaction

Plate 5

Plate 6: Symptoms in susceptible reaction on leaf lamina

Plate 6

Plate 7: Diseased leaves showing the rating scale for severity (from left to right 1,3,5,7 and 9 respectively)

Plate 7

RESULTS

CHAPTER IV

RESULTS

4.1 MORPHOLOGICAL VARIABILITIES

Morphology of the conidia, appressoria, setae, sclerotia and acervulus of 12 isolates of *Colletotrichum graminicola* were studied to find the variation among the isolates.

4.1.1 Conidia

Variation was recorded in respect to shape and size of the conidia. The length of conidia of the different isolates varied from 22.27 to 27.29 µm and breadth from 3.14 to 3.96 µm (Table 3). The isolate Cg 138 showed minimum length (22.27 µm) compared to maximum (27.29µm) in isolate Cg 227 (Plate 8 and 9). The breadth of conidia varied from 3.14 µm in Cg 118 to 3.96 µm in Cg 226 (Fig.1). Based on the length and breadth of conidia the isolates could be differentiated into six groups.

The shape of the conidia of all isolates was falcate. However, Cg 20, Cg 75, Cg 118, Cg 130, Cg 138, Cg 150, Cg 158, Cg 226 and Cg 226-3 produced spindle-shaped conidia (Table 6; Plate 10).

4.1.2 Appressoria

The culture of the pathogen on OMA medium after 10 days of incubation was recorded for the production and morphology of appressoria. All the isolates produced appressoria, except Cg 94 and Cg 150. These

Table 3: Variation in size* of conidia among 12 isolates of *Colletotrichum graminicola* grown on OMA medium 10 days after incubation

Isolates designation	Length (µm)		Breadth (µm)	
	Range	Mean**	Range	Mean**
Cg 20	17-26	22.40 ± 2.02^{a}	3-4	3.49 ± 0.51^{b}
Cg 75	19-29	25.27 ± 2.10^{d}	3-4	3.71 ± 0.46^{d}
Cg 94	18-27	23.71 ± 1.95^{b}	3-4	3.61 ± 0.49^{c}
Cg 118	18-36	24.47 ± 3.37^{c}	3-4	3.14 ± 0.35^{a}
Cg 130	18-29	25.53 ± 1.96^{d}	3-4	3.49 ± 0.50^{b}
Cg 138	17-27	22.27 ± 2.04^{a}	3-4	3.47 ± 0.50^{b}
Cg 150	15-31	23.76 ± 2.59^{bc}	3-4	3.55 ± 0.50^{bc}
Cg 158	20-29	25.69 ± 1.84^{d}	3-5	3.74 ± 0.491^{d}
Cg 226	21-28	24.20 ± 1.61^{c}	3-5	3.96 ± 0.29^{f}
Cg 226-3	18-33	26.45 ± 3.00^{e}	3-5	3.88 ± 0.39^{e}
Cg 227	22-32	27.29 ± 2.00^{f}	3-4	3.88 ± 0.33^{e}
Cg 227-3	21-33	26.86 ± 2.72^{e}	3-5	3.84 ± 0.43^{e}
SEM		± 0.472		± 0.067
CV (%)		6.59		6.39

* Mean of 50 conidia

** Means with the same letter are not significantly different

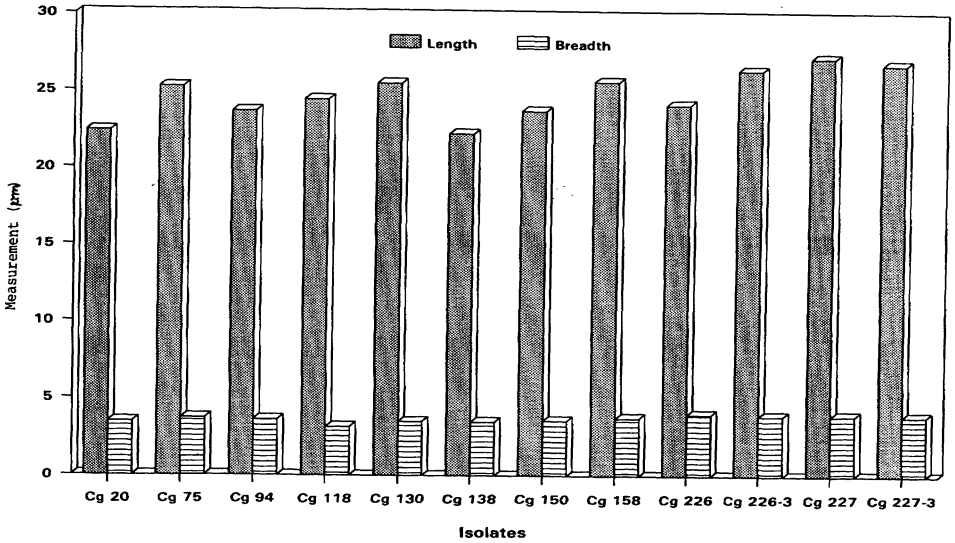


Fig.1: Variation in size of conidia among 12 isolates of C. graminicola grown on OMA medium 10 days after incubation

two isolates were unable to produce appressoria even after 20 days of incubation.

Significant differences were recorded among the isolates of *C. graminicola* regarding the length and breadth of appressoria (Table 4). Appressoria of Cg 226 was shortest (14.50 µm) and that of isolate Cg 20 the longest (20.00 µm). Isolate Cg 118 showed the least breadth (7.90 µm) and isolate Cg 130 had maximum breadth (11.40 µm) (Fig.2). However, isolates Cg 130 and Cg 20 produced abundant appressoria (Plate 11).

The shape of appressoria in most of the isolates was oval, except Cg 138, which produced both lobed and oval appressoria (Plate 12). An interesting aspect was observed in case of the position of the appresoria. Production of appressoria was terminal in all the isolates, except Cg 130 where it was terminal as well as intercalary (Plate 13). Both longitudinal and transverse septation was another distinguishing character found in appressoria of Cg 130 and Cg 20 (Plates 14 and 15), whereas all other isolates produced only aseptate appressoria.

4.1.3 Setae

The number of setae per acervulus and their size were measured to find the differences among the 12 isolates of *C. graminicola* (Table 5). All the isolates produced setae on OMA medium 10 days after incubation except, Cg 118 (Plate 16). When Cg 118 was further incubated for 10 days it produced setae sparsely, shorter in length and breadth, and the colour ranged from yellow to light brown.

Table 4: Variation in size* of appressoria among 12 isolates of *Colletotrichum graminicola* grown on OMA medium 10 days after incubation

Isolates designation	Length (µm)		Breadth (µm)	
	Range	Mean***	Range	Mean***
Cg 20	17-22	20.00 ± 1.886^{f}	8-11	9.60 ± 1.075^{cd}
Cg 75	13-21	16.60 ± 2.459^{c}	7-12	9.50 ± 1.581^{cd}
Cg 94**	-	-	-	-
Cg 118	12-22	16.40 ± 3.627^{c}	5-12	7.90 ± 2.079^{a}
Cg 130	16-22	18.60 ± 2.066^{e}	10-14	11.40 ± 1.174^{f}
Cg 138	14-22	16.60 ± 2.675^{c}	8-12	10.50 ± 1.269^{e}
Cg 150**	-	-	-	-
Cg 158	14-22	17.80 ± 2.394^{d}	8-12	11.00 ± 1.155^{f}
Cg 226	12-18	14.50 ± 2.014^{a}	7-12	9.30 ± 1.337^{c}
Cg 226-3	12-20	14.60 ± 2.366^{a}	7-10	8.90 ± 1.287^{b}
Cg 227	13-20	15.60 ± 2.271^{b}	7-11	9.00 ± 1.414^{bc}
Cg 227-3	15-18	16.60 ± 1.075^{c}	9-12	9.80 ± 1.033^{d}
SEM		±0.54		±0.33
CV (%)		10.21		10.77

* Mean of 10 appressoria

** No production of appressoria

*** Means with the same letter are not significantly different

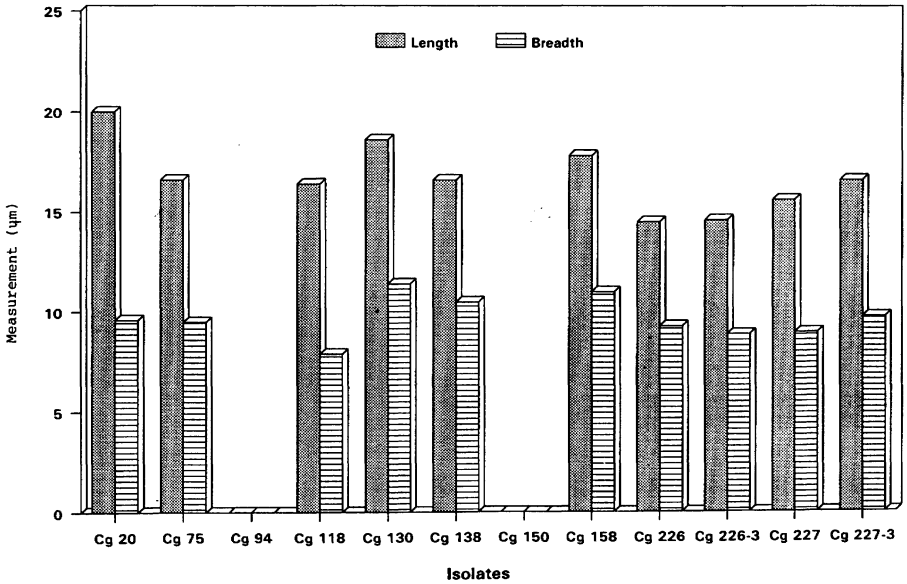


Fig.2: Variation in size of appressoria among 12 isolates of C. graminicola grown on OMA medium 10 days after incubation

Maximum number (mean 27.6) of setae per acervulus were observed in isolate Cg 20 (Plate 17) and the minimum in isolate Cg 227-3 (mean 0.5), (Table 5). Significant variations in length and breadth of setae were found among all the isolates. Isolate Cg 226-3 produced the longest (111.80 μm) setae compared to the shortest setae (76.48 μm) in isolate Cg 130, whereas setae in isolates, Cg 138 and Cg 226 were broader (4.86 μm) than all the isolates and the least breadth (4.41 μm) was found in isolate Cg 20 (Table 5).

Although all the isolates produced typical setae i.e., brown, smooth, septate, tapered to the apices; isolate Cg 150 produced a different type of setae along with normal one which were shorter in length and blunt at tip with hyaline apex. It was also observed that the setae produced conidia at the apex, resembling that of a conidiophore (Plates 18 and 19). Indistinct acervulus with branched setae also was observed in isolate Cg 226-3 (Plate 20).

4.1.4 Acervulus

All the isolates produced acervuli on OMA medium 10 days after incubation but differences were found regarding their form, colour and distinction. Isolates Cg 130 and Cg 138 produced acervulus as yellowish to light brown masses with or without setae, irregular in shape and the conidiogenous cells were not clearly found. This type of acervuli were designated as "indistinct" (Table 6 and Plate 20). In other isolates, fungus produced acervuli generally dark brown in colour, circular or semicircular in shape and conidiogenous cells were found distinctly, which also became

Table 5: Variation in number* and size** of the setae among 12 isolates of *Colletotrichum graminicola* grown on OMA medium 10 days after incubation

Isolates designation	No.of setae/acervulus		Size of the setae			
			Length (µm)		Breadth (µm)	
	Range	Mean****	Range	Mean****	Range	Mean****
Cg 20	21-34	27.60±3.92^{f}	62.0-136.4	97.76±16.41^{e}	2.48-4.96	4.41±1.04^{a}
Cg 75	6-13	9.70±2.16^{cd}	59.5-151.3	91.81±18.79^{d}	2.48-4.96	4.76±0.68^{b}
Cg 94	9-20	14.00±3.33^{e}	57.0-121.5	81.99±16.48^{b}	2.48-4.96	4.76±0.68^{b}
Cg 118***	-	-	-	-	-	-
Cg 130	0-2	0.80±0.79^{ab}	54.6-183.5	76.48±21.47^{a}	2.48-4.96	4.81±6.60^{c}
Cg 138	0-3	1.13±0.95^{ab}	54.6-173.6	87.20±22.48^{c}	2.48-4.96	4.86±0.49^{d}
Cg 150	5-10	7.20±1.48^{c}	69.4-138.9	93.64±17.48^{d}	2.48-4.96	4.81±0.60^{c}
Cg 158	9-14	11.20±1.62^{d}	69.4-171.1	91.00±26.77^{d}	2.48-4.96	4.81±0.56^{c}
Cg 226	2-5	3.00±1.33^{b}	74.4-161.2	106.81±22.63^{g}	2.48-4.96	4.86±0.49^{d}
Cg 226-3	7-10	8.60±0.97^{c}	74.4-151.3	111.80±17.88^{h}	2.48-4.96	4.81±0.60^{c}
Cg 227	0-3	1.40±0.84^{ab}	70.4-143.8	109.49±18.00^{gh}	2.48-4.96	4.81±0.60^{c}
Cg 227-3	0-1	0.50±0.53^{a}	74.4-176.6	102.68±18.00^{f}	2.48-4.96	4.81±0.60^{c}
SEM		±2.32		±3.409		±0.037
CV (%)		113.05		11.81		2.59

* Mean of 10 randomly selected acervulus
** Mean of 50 setae
*** Setae were not produced
**** Means with the same letter are not significantly different

blue along with conidia when stained with 0.1 per cent lactophenol-cotton blue. This type of acervuli were designated as "distinct". Isolates Cg 158, Cg 226 and Cg 226-3 produced both distinct and indistinct types of acervuli.

4.1.5 Sclerotia

The presence or absence of sclerotia was observed in 10 day-old culture on OMA medium. It was found that isolates Cg 94, Cg 118, Cg 150, Cg 226-3 and Cg 227-3 produced matured sclerotia (Plate 21) and isolates Cg 75, Cg138, Cg 226 produced young, forming or immature, sclerotia (Plate 22) in the same age of cultures whereas in other isolates (Cg 20, Cg 130, Cg 158 and Cg 227) sclerotia were absent (Table 6). Further when these cultures were observed 20 days after incubation, isolate Cg 20 produced mature sclerotia, but rest did not produce sclerotia.

4.2 CULTURAL VARIABILITY

Cultural variability was recorded by comparing the rate of colony growth characters and sporulation of all isolates on OMA medium 10 days after incubation. Twelve isolates differed distinctly in colony morphology, colony diameter,colour of the mycelium (Plate 23) and sporulation.

4.2.1 Colony growth

Colony of the pathogen on OMA medium varied widely in respect to colony diameter as well as growth pattern. Colony diameter ranged between 52.33 mm (Cg 227) and 73.67 mm (Cg 130). Two bulk cultures

Table 6: Variation in morphological characteristics of 12 isolates of *Colletotrichum graminicola* 10 days after incubation on OMA medium at 25°C

Isolates	Colony	Colour	Acervuli	Appressoria	Sclerotia	Conidial dimorphism	Remarks
Cg 20	Wooly, raised, margin distinct	Grey at centre, white at margin	D	+[1]	-[2]	+	Septate appressoria found
Cg 75	Wooly, raised margin distinct	Greyish white centre, white at margin	D	+	+[3]	+	
Cg 94	Wooly, submerged, margin distinct	Greyish with salmon orange background	D	-	+	-	Sectoring appeared in colony
Cg 118	Wooly, submerged, nodular, margin diffused	Grey with salmon orange background	D	+	+	+	
Cg 130	Wooly, submerged, margin diffused	Greyish white background salmon orange	I	+[1]	-	+	Appressoria produced in intermediate position of hyphae in considerable number, septate appressoria found
Cg 138	Wooly, submerged, margin diffused	Greyish white with salmon orange background	I	+	+[3]	+	Lobed appressoria produced

Contd..

Contd..

Isolates	Colony	Colour	Acervuli	Appressoria	Sclerotia	Conidial dimorphism	Remarks
Cg 150	Wooly, raised, margin distinct	Whitish grey	D	-	+	+	Conidia bearing setae observed
Cg 158	Wooly, raised, margin diffused	Whitish grey	D/I	+	-	+	
Cg 226	Felty, submerged, margin diffused	White	D	+	+[3]	+	
Cg 226-3	Felty, raised, margin distinct	White	D/I	+	+	+	Appressoria very rare
Cg 227	Felty, submerged, margin distinct	Greyish white	D	+	-	-	
Cg 227-3	Felty, submerged margin distinct	Greyish white	D/I	+	+	-	Sectoring appeared in colony

D - Distinct
I - indistinct
\+ - present
\- - absent

1 - abundant appressoria produced
2 - sclerotia formed at 20 days after incubation
3 - Young sclerotia found

(Cg 226 and Cg 227) and their monoconidial cultures (Cg 226-3 and Cg 227-3) were not significantly different in colony measurement (Table 7).

Isolates Cg 227, Cg 226-3 and Cg 227-3 produced felty submerged growth whereas all other isolates produced either wooly (Cg 20, Cg 75, Cg 130, Cg 150, Cg 158) or submerged wooly (Cg 94, Cg 118, Cg 138, Cg 226) colony. Only few isolates produced diffused margin of the colony (Cg 94, Cg 118, Cg 130, Cg 138) and others showed a distinct margin of the colony (Table 6, Plate 23). Sectoring in colony was observed in isolates Cg 94 and Cg 227-3.

Colour of the colony varied from white to whitish grey and to salmon orange (Table 7). Two isolates Cg 118 and Cg 138 produced submerged greyish white colony with salmon orange background.

4.2.2 Sporulation

Sporulation on OMA medium 10 days after incubation was significantly different in majority of isolates. Production of spores per mm^2 of the colony varied from 2.90×10^4 to 51.97×10^4 in case of isolate Cg 130 and Cg 227-3 respectively (Table 8).

4.3 PATHOGENIC VARIABILITY

Symptoms on inoculated leaves developed ranging from resistant (no infection or chlorotic lesion) to susceptible (circular or elliptical necrotic lesions upto 5 mm in diameter). Lesions appeared on both surfaces of the leaves. The lesions varied from tan to orange-red to black depending on

Table 7: Variation in colony growth* of 12 isolates of *Colletotrichum graminicola* on OMA medium 10 days after incubation at 25°C

Isolates designation	Range (mm)	Mean diameter** (mm)
Cg 20	53-58	56.00 ± 2.646^{ab}
Cg 75	56.71	64.67 ± 7.67^{c}
Cg 94	56-59	57.33 ± 1.53^{ab}
Cg 118	57-60	58.33 ± 1.53^{b}
Cg 130	73.75	73.67 ± 1.56^{d}
Cg 138	64-71	68.00 ± 3.61^{c}
Cg 150	63-70	65.67 ± 3.79^{c}
Cg 158	59-71	66.67 ± 6.66^{c}
Cg 226	53-60	56.67 ± 3.51^{ab}
Cg 226-3	53-54	53.67 ± 0.58^{ab}
Cg 227	50-56	52.33 ± 3.22^{a}
Cg 227-3	49-63	54.33 ± 7.57^{ab}
SEM		± 5.20
CV (%)		29.74

* Mean of 3 replications

** Means with the same letter are not significantly different

Table 8: Variation in sporulation* (per mm^2 of the colony) of 12 isolates of *Colletotrichum graminicola* on oat meal agar 10 days after incubation at $25^{o}C$

Isolates designation	Range ($x10^4/mm^2$)	Mean ($x10^4/mm^2$)**
Cg 20	5.60-6.80	6.17 ± 0.408^{b}
Cg 75	4.40-8.40	6.73 ± 1.547^{b}
Cg 94	2.20-4.20	3.23 ± 0.709^{a}
Cg 118	7.60-33.60	15.417 ± 9.651^{d}
Cg 130	2.20-3.80	2.90 ± 0.576^{a}
Cg 138	20.60-24.80	22.67 ± 1.751^{e}
Cg 150	3.20-4.20	3.70 ± 0.374^{a}
Cg 158	3.20-5.20	4.33 ± 0.776^{ab}
Cg 226	15.60-33.60	24.80 ± 5.808^{e}
Cg 226-3	2.00-4.40	3.13 ± 1.025^{a}
Cg 227	9.60-12.80	11.03 ± 1.268^{c}
Cg 227-3	49.80-54.00	51.97 ± 1.835^{f}
SEM		± 2.25
CV (%)		5.48

* Mean of 3 replications

** Means with the same letter are not significantly different

the sorghum genotypes. Among necrotic lesions in some host-isolate interactions black specks, which are part of the acervulus, were observed at the centre of lesions indicating susceptible reaction. The hypersensitive reaction was characterized by small necrotic spots without black specks (acervulus).

Twelve isolates varied significantly in respect to virulence (disease reaction), aggressiveness (disease severity) and latent period.

4.3.1 Latent period

The latent period of isolates varied from 2.75 to 4.59 days (Table 9) in all host-isolate interactions. The isolate Cg 226 had the shortest mean latent period of 3.6 days across six sorghum lines while Cg 130 had the longest (4.11 days). Among the sorghum lines, longest mean latent period (4.38 days) across the isolates was on A 2267-2, followed by 4.31 days on IRAT 204 and the shortest was on IS 3758. There was no significant difference between latent periods of monoconidial cultures (Cg 226-3 and Cg 227-3) with their respective parent isolates across the host lines.

Highly significant ($P<0.001$) effects of isolates and host genotypes were observed for latent period (Table 13).

4.3.2 Virulence

All the 12 isolates were avirulent on A2267-2 and IRAT 204, but 6 and 9 of them produced chlorotic flecks on A2267-2 and IRAT 204,

Table 9: Variation in latent period* (days) of 12 isolates of *Colletotrichum graminicola* on six sorghum lines

Isolate designation	Sorghum lines						
	A 2267-2	IRAT 204	IS 8354	IS 3758	IS 3089	IS 18442	Mean***
Cg 20	4.29	4.27	3.59	3.50	3.70	3.35	3.78^{ab}
Cg 75	4.50	4.31	3.55	2.95	3.50	3.80	3.76^{ab}
Cg 94	-**	-	3.90	3.40	4.45	4.00	3.94^{b}
Cg 118	-	-	4.10	3.50	4.49	4.05	4.04^{b}
Cg 130	4.52	4.33	3.80	3.60	4.30	4.10	4.11^{b}
Cg 138	-	-	3.90	3.70	3.76	3.65	3.75^{ab}
Cg 150	4.44	4.33	3.80	3.65	3.95	3.55	3.95^{b}
Cg 158	-	4.33	3.15	3.30	3.65	3.90	3.66^{a}
Cg 226	4.13	4.23	3.50	2.75	3.55	3.45	3.60^{a}
Cg 226-3	-	-	3.80	3.15	3.80	4.00	3.69^{a}
Cg 227	-	4.08	4.10	3.10	3.90	3.60	3.76^{ab}
Cg 227-3	-	4.59	4.00	3.80	3.65	3.95	4.00^{b}
Mean***	4.38^{c}	4.31^{c}	3.77^{b}	3.37^{a}	3.89^{b}	3.78^{b}	

LSD (P<0.05) for isolate means = 0.24
LSD (P<0.05) for sorghum lines means = 0.17
LSD (P<0.05) for isolate x sorghum lines means = 0.58

* Mean of 2 experimental runs
** No flecks or lesion
*** Means with the same letter are not significantly different

respectively (Table 9). Majority of these isolates were virulent on IS 8354, IS 3758, IS 3089 and IS 18442. Among these 4 host genotypes, IS 3089 and IS 18442 showed susceptible reaction for all 12 isolates and other two genotypes showed differential reactions. Genotype IS 8354 was moderately resistant to Cg 118 and Cg 138 and susceptible for all other isolates. On the otherhand, IS 3758 showed susceptible reaction to Cg 158, Cg 226, Cg 226-3 and Cg 227, but moderately resistant to the remaining isolates (Table 10).

Among the two monoconidial cultures Cg 226-3 showed similar disease reaction like its parent isolate (Cg 226) but Cg 227-3 showed moderately resistant reaction on IS 3758 unlike its parent isolate (Cg 227). In case of Cg 226, although monoconidial culture (Cg 226-3) gave resistant reaction on A 2267-2 and IRAT 204 it was unable to produce chlorotic flecks unlike its parent isolate.

Considering the virulence of different host- isolate interactions, isolates of *C. graminicola* could be differentiated into three distinct groups - GR1 (Cg 118 and Cg 138), GR2 (Cg 158, Cg 226, Cg 226-3 and Cg 227), and GR3 (Cg 20, Cg 75, Cg 94, Cg 130, Cg 150, Cg 227-3). Among these three groups, the GR1 (Cg 118 and Cg 138) was the least virulent infecting only two of six sorghum lines, whereas the GR2 (Cg 158, Cg 226, Cg 226-3 and Cg 227) was the most virulent infecting four of the six sorghum lines.

Table 10: Virulence (disease reaction) of 12 isolates of *Colletotrichum graminicola* on six sorghum lines

Isolate designation	Sorghum lines					
	A 2267-2	IRAT 204	IS 8354	IS 3758	IS 3089	IS 18442
Cg 20	R	R	S	MR	S	S
Cg 75	R	R	S	MR	S	S
Cg 94	R	R	S	MR	S	S
Cg 118	R	R	MR	MR	S	S
Cg 130	R	R	S	MR	S	S
Cg 138	R	R	MR	MR	S	S
Cg 150	R	R	S	MR	S	S
Cg 158	R	R	S	S	S	S
Cg 226	R	R	S	S	S	S
Cg 226-3	R	R	S	S	S	S
Cg 227	R	R	S	S	S	S
Cg 227-3	R	R	S	MR	S	S

4.3.3 Aggressiveness

All isolates were more aggressive (2.40-6.10) on IS 3758, IS 38354, IS 3089 and IS 18442 and least aggressive (<1.8 severity) on A 2267-2 and IRAT 204 (Table 11; Plates 24 and 25). Mean aggressiveness of isolates across six sorghum lines was highest in Cg 226 (3.71) and lowest in Cg 118 (2.25). Significant ($P<0.05$) effects of isolates and sorghum lines and their interactions were observed for aggressiveness (Table 13). Monoconidial isolate of Cg 226 i.e., Cg 226-3 was also significantly different from its parent isolate considering mean aggressiveness (Table 11). However, similar result was not observed for Cg 227 and its monoconidial derived isolate Cg 227-3.

Twelve isolates of *C. graminicola* could be divided into four groups based on disease severity on six sorghum lines where Cg 118, Cg 150 and Cg 226 formed into three individual groups and the remaining isolates were included into Group 4. Isolate Cg 150 was intermediate, whereas the remaining three groups were significantly different (Table 11).

4.3.4 Virulence index

Variation was observed in virulence index of different isolates (Table 12). Mean virulence index was highest in Cg 226 (3.00) and lowest in Cg 130 (1.64). Means were also significantly different among both isolates and genotypes. Virulence index was maximum for IS 18442 and minimum for A 2267-2. There was no significant difference between parent isolates and their monoconidial isolates. According to virulence index isolates could

Table 11: Aggressiveness* (disease severity on a 1-9 scale) of 12 isolates of *Colletotrichum graminicola* on six sorghum lines

Isolate designation	Sorghum lines						
	A 2267-2	IRAT 204	IS 8354	IS 3758	IS 3089	IS 18442	Mean***
Cg 20	1.60	1.90	2.63	3.60	3.13	3.40	2.71^{b}
Cg 75	1.59	1.70	4.05	3.15	3.25	3.25	2.83^{b}
Cg 94	1.00**	1.00	3.00	3.90	4.00	4.25	2.86^{b}
Cg 118	1.00	1.00	2.60	3.10	3.10	2.70	2.25^{a}
Cg 130	1.55	1.70	2.40	3.90	3.15	4.05	2.79^{b}
Cg 138	1.00	1.00	2.65	3.75	5.21	2.55	2.69^{b}
Cg 150	1.50	1.55	3.00	3.30	2.75	3.50	2.60^{ab}
Cg 158	1.00	1.15	3.70	3.25	4.70	3.45	2.88^{b}
Cg 226	1.65	1.80	4.05	5.10	3.58	6.10	3.71^{c}
Cg 226-3	1.00	1.00	3.50	3.25	3.05	4.80	2.87^{b}
Cg 227	1.00	1.70	2.60	3.50	2.95	4.60	2.73^{b}
Cg 227-3	1.00	1.80	4.50	4.50	2.35	3.40	2.93^{b}
Mean***	1.24^{a}	1.44^{a}	3.22^{b}	3.69^{c}	3.43^{bc}	3.84^{c}	

LSD (P<0.05) for isolate means = 0.36
LSD (P<0.05) for sorghum line means = 0.26
LSD (P<0.05) for isolate x sorghum line means = 0.88

* Mean of 2 experimental runs
** No flecks or lesion
*** Means with the same letter are not significantly different

be divided into five groups — three groups varied distinctly to each other and two groups were intermediate.

Correlation coefficient of latent period, disease reaction, aggressiveness and virulence index (Table 14) indicated that latent period was negatively correlated with aggressiveness and virulence index whereas disease reaction, aggressiveness and virulence index were positively correlated to each other significantly ($P<0.001$). Correlation coefficient ($r=0.90$) of aggressiveness to virulence index was highly significant ($P<0.001$) compared with the correlation coefficient ($r=0.70$) of virulence and latent period ($r=0.68$).

According to dendrograms developed based on aggressiveness and virulence index the isolates were divided into three and four groups, respectively (Fig.3 and 4). Although in both cases isolates in a particular group were different but Cg 226 showed wider diversity than others.

4.4 MOLECULAR VARIABILITY

Pattern of bands on the agarose gel indicated DNA polymorphism among the isolates. Comparison showed that each isolate was different in length of DNA fragments amplified (position of each band) and in the number of fragments (number of band per isolate), indicating a high degree of variability in the fungus.

The primer OPA8 (5'GTGACGTAGG3') produced bands in 15 different positions with size ranging between 0.2 kb (200 bp) and 2.0 kb (2000 bp)

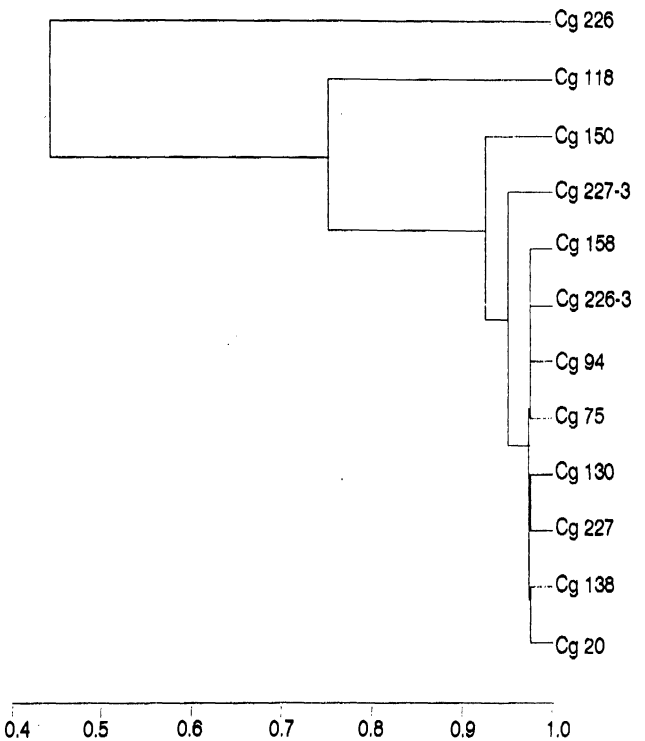
Table 12: Virulence index* of 12 isolates of *Colletotrichum graminicola* on six sorghum lines

Isolate designation	Sorghum lines						
	A 2267-2	IRAT 204	IS 8354	IS 3758	IS 3089	IS 18442	Mean***
Cg 20	0.38	0.45	2.40	2.09	2.38	3.03	1.79^{ab}
Cg 75	0.35	0.40	3.51	2.39	2.65	2.67	2.00^{ab}
Cg 94	-**	-	2.32	2.35	2.72	3.24	2.66^{c}
Cg 118	-	-	1.27	1.78	1.79	1.99	1.70^{a}
Cg 130	0.34	0.39	1.90	2.17	1.99	3.03	1.64^{a}
Cg 138	-	-	1.65	2.08	4.52	2.03	2.57^{c}
Cg 150	0.34	0.37	2.52	1.89	1.86	3.12	1.68^{a}
Cg 158	-	0.37	4.19	2.81	3.68	2.78	2.77^{cd}
Cg 226	0.40	0.44	3.49	4.96	2.91	5.77	3.00^{d}
Cg 226-3	-	-	2.75	2.54	2.16	3.63	2.77^{cd}
Cg 227	-	0.41	1.91	2.32	2.35	4.29	2.26^{bc}
Cg 227-3	-	0.39	3.36	2.38	1.79	2.75	2.13^{b}
Mean***	0.362^{a}	0.40^{a}	2.61^{b}	2.48^{b}	2.57^{b}	3.19^{c}	

LSD (P<0.05) for isolate means = 0.37
LSD (P<0.05) for sorghum line means = 0.27
LSD (P<0.05) for isolate x sorghum line means = 0.92

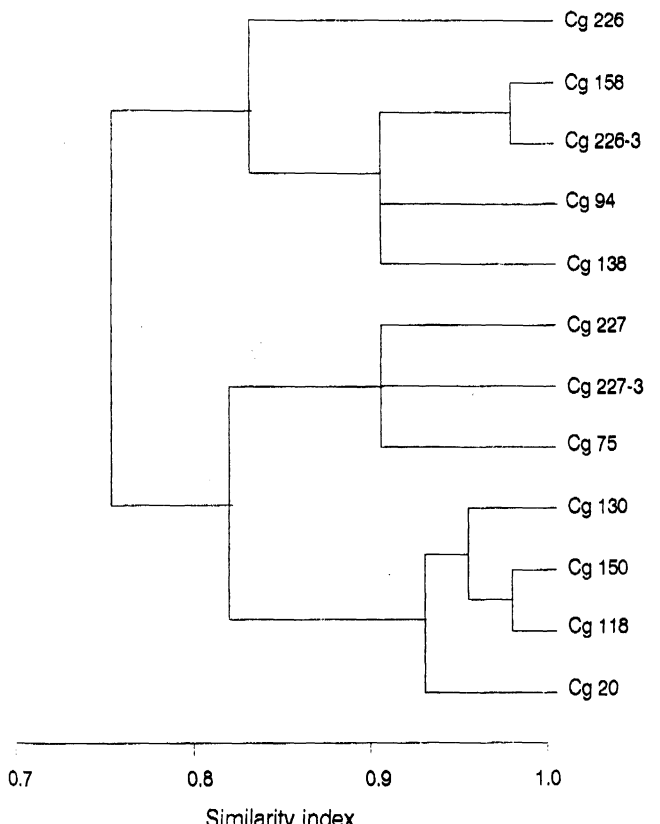
* Mean of 2 experimental runs
** No flecks or lesion
*** Means with the same letter are not significantly different

Figure 3



Dendrogram based on aggressiveness of 12 isolates of *Colletotrichum graminicola* on six sorghum lines

Figure 4



Dendrogram based on virulence index of 12 isolates of *Colletotrichum graminicola* on six sorghum lines

Table 13: Analysis of variance for latent period (LP), virulence index (VI) and aggresiveness (AG)

Sources of variation	Mean square			Mean square
	df	LP	VI	df	AG
Experiment (E)	1	10.42***	10.394***	1	48.68***
Isolates (I)	11	0.63***	5.94***	11	2.67***
Sorghum lines (G)	5	4.68***	45.19***	5	64.59***
E x I	11	0.37**	2.68***	11	0.89***
E x G	5	1.07***	9.98***	5	9.30***
I x G	44	0.23***	1.71***	55	1.67***
E x I x G	43	0.26**	0.81***	55	0.93***
Error	120	0.14	0.30	144	0.20
Total	240				

* Significant at P<0.05
** Significant at P<0.01
*** Significant at P<0.001

Table 14: Correlation coefficient among latent period (LP), disease reaction (DR), disease severity (DS) and virulence index (VI)

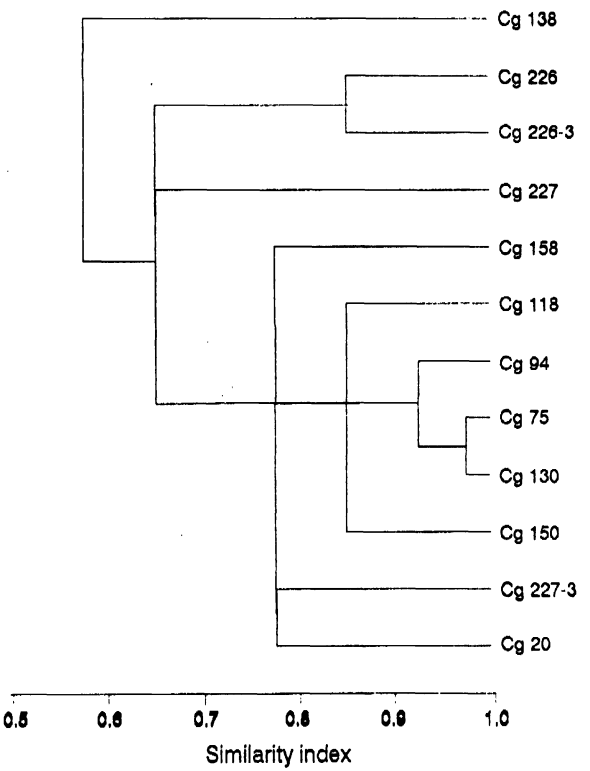
	DR	DS	VI
LP	-0.374***	-0.475***	-0.675***
DR		0.697***	0.696***
DS			0.900***

*** Significant at P<0.001

(Plate 27). It was found from the similarity index that 57.5 per cent of the DNA amplified bands were common to all isolates, while 42.5 per cent were polymorphic (i.e., present in some isolates but absent in others). Isolate Cg 138 showed maximum polymorphism regarding position of bands and the first band size was of 1.2 Kb. Among two monocoindial isolates Cg 226-3 produced bands like its parent isolate Cg 226 according to number and position but in Cg 227-3 number and position of the band was different from its parent isolate Cg 227. Least number of bands were produced by Cg 20 when compared to other isolates. Presence or absence of a band specific to more than one isolate were considered phylogenetically informative as they were useful for clustering relationships. Polymorphism, that was unique to one isolate is noninformative but considered for estimation of divergence or genetic distance value.

Cluster analysis performed with the GENSTAT program produced a tree from maximum to minimum similarity among isolates. From the dendrogram based on this analysis isolates could be classified into four groups (Fig.5). The first group consisting of five isolates (i.e., Cg 150, Cg 130, Cg 75, Cg 94 and Cg 118) was subdivided into three sub groups where Cg 75 and Cg 130 included in subgroup I; Cg 94 alone formed the subgroup II; and Cg 118 and Cg 150 subgroup III. Isolates Cg 20, Cg 227-3 and Cg 158 formed the second group without any subgroup. Other three isolates Cg 226, Cg 226-3 and Cg 227 were included in group three where Cg 226 and Cg 226-3 formed subgroup I and Cg 227 in sub group II. The fourth group consisted of only one isolate Cg 138.

Figure 5



Dendrogram based on RAPD analysis of 12 isolates of ***Colletotrichum graminicola*** using OPA8 primer

Interestingly, the midrib isolate Cg 227 and its monoconidial isolate Cg 227-3 were clustered in separate groups having 65 per cent similarity between them. However, another parent isolate Cg 226 and its monoconidial isolate Cg 226-3 were in the same subgroup with 85 per cent similarity.

It was clear from the RAPD analysis that there was no significance of geographical regions (4 states of India) in degree of polymorphism. Moreover, isolates collected from the same state with or without same symptoms or plant parts showed distinct polymorphism forming separate groups.

Dendrogram based on RAPD analysis and virulence index could not be compared to each other. RAPD analysis showed more diversity than virulence analysis and diversity of an individual isolate from others was different in the two analysis. Although isolates were clustered into four groups in both the analysis, isolates included in a particular group were different.

Plate 8: Smallest conidia of Cg 138 isolate of *C. graminicola* (400x)

Plate 9: Largest conidia of Cg 227 isolate of *C. graminicola* (400x)

Plate 8

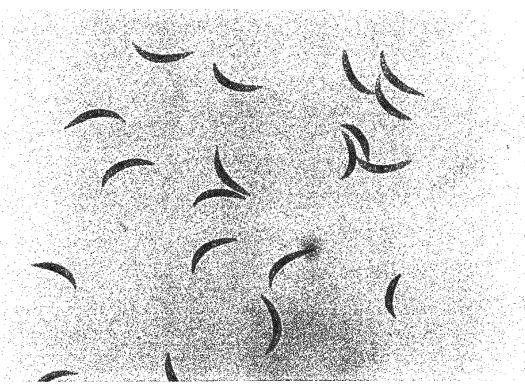


Plate 9

Plate 10: Straight (arrows) and falcate conidia produced by Cg 138 isolate of *C. graminicola* (100x)

Plate 10

Plate 11: Typical appressoria produced by Cg 20 isolate of *C. graminicola* 10 days after incubation on OMA medium (400x)

Plate 12: Lobed appressoria (arrows) produced by Cg 138 isolate of *C. graminicola* 10 days after incubation on OMA medium (400x)

Plate 11

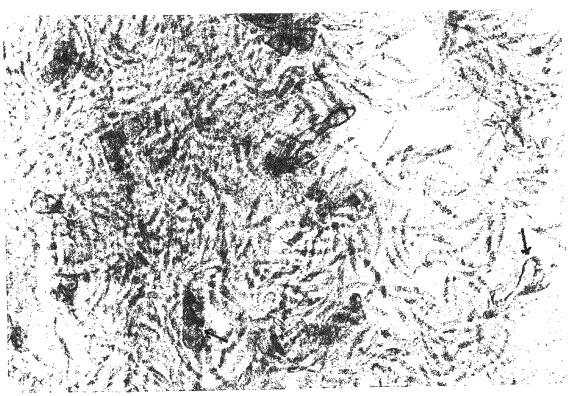


Plate 12

Plate 13: Production of terminal (arrowhead) and intercalary (arrows) appressoria in Cg 130 of *C. graminicola* (400x)

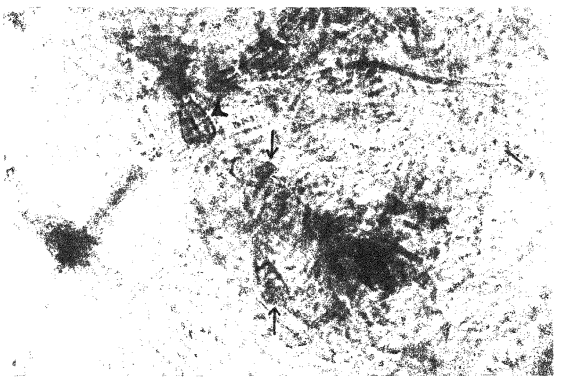


Plate 13

Plate 14: Septate appressoria (arrow) of Cg 130 isolate of *C. graminicola* (400x)

Plate 15: Septate appressoria (arrow) of Cg 20 isolate of *C. graminicola* (1000x)

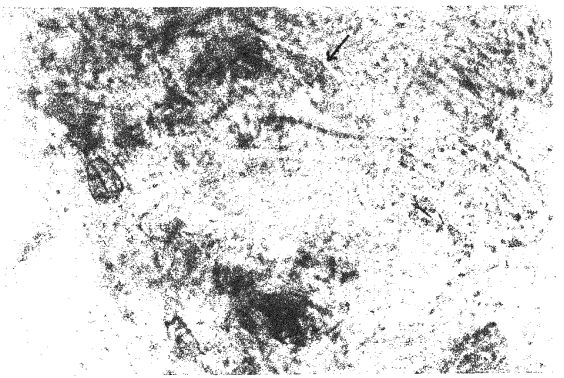


Plate 14

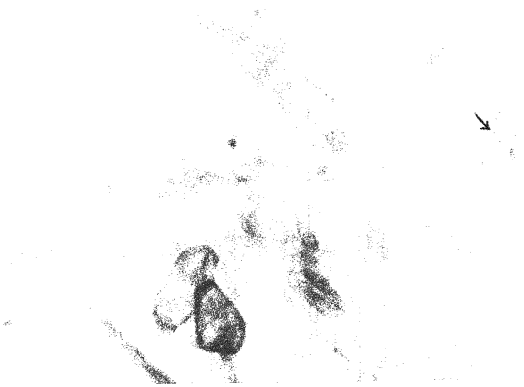


Plate 15

Plate16: Acervulus without setae produced in Cg 118 isolate of *C. graminicola* 10 days after incubation (400x)

Plate 17: Numerous setae per acervulus produced in Cg 20 isolate of *C. graminicola* 10 days after incubation (200x)

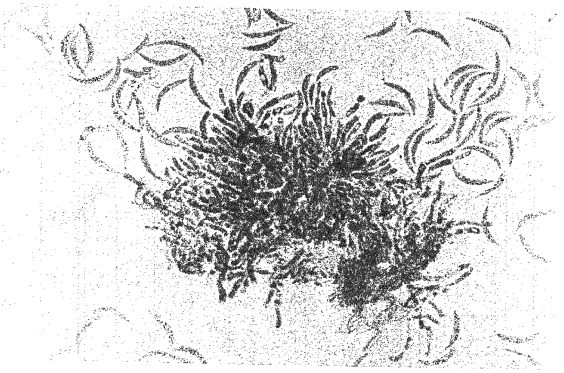


Plate 16

Plate 17

Plate 18: Conidia produced on setae in Cg 150 isolate of *C. graminicola* 10 days after incubation (1000x)

Plate 19: Conidia-bearing setae and conidiospore emerged from same position of the hyphae in a mature acervulus of Cg 150 isolate of *C. graminicola* (1000x)

Plate 18

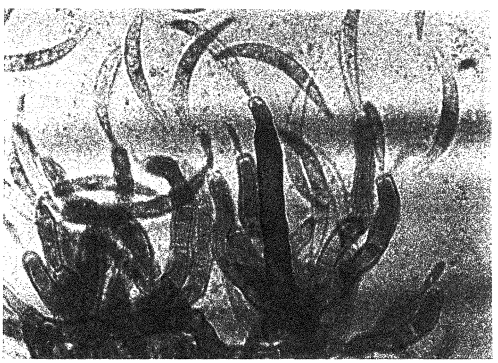


Plate 19

Plate 20: Indistinct acervulus with branched setae in Cg 226-3 isolate of *C. graminicola* 10 days after incubation (400x)

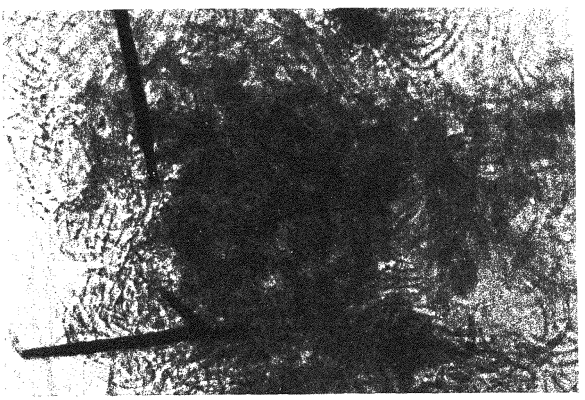


Plate 20

Plate 21: Mature sclerotia produced by Cg 150 isolate of *C. graminicola* 10 days after incubation (400x)

Plate 22: Immature sclerotia produced by Cg 75 isolate of *C. graminicola* 10 days after incubation on OMA medium (400x)

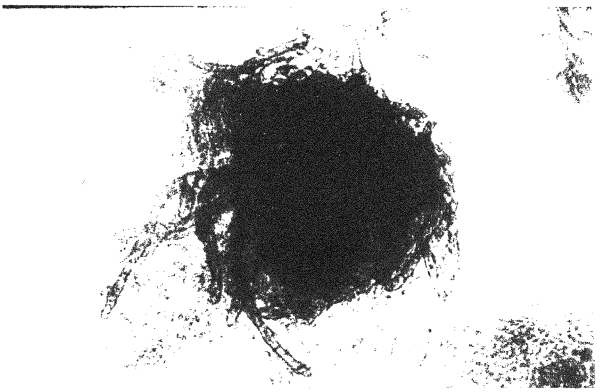


Plate 21

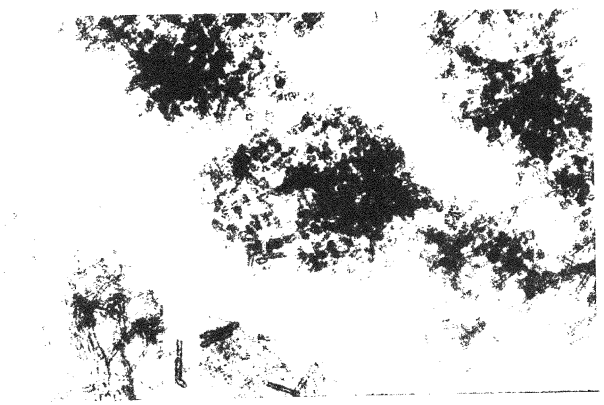


Plate 22

Plate 23: Variation in growth characteristics of 12 isolates of *C. graminicola* grown on OMA medium 10 days after incubation

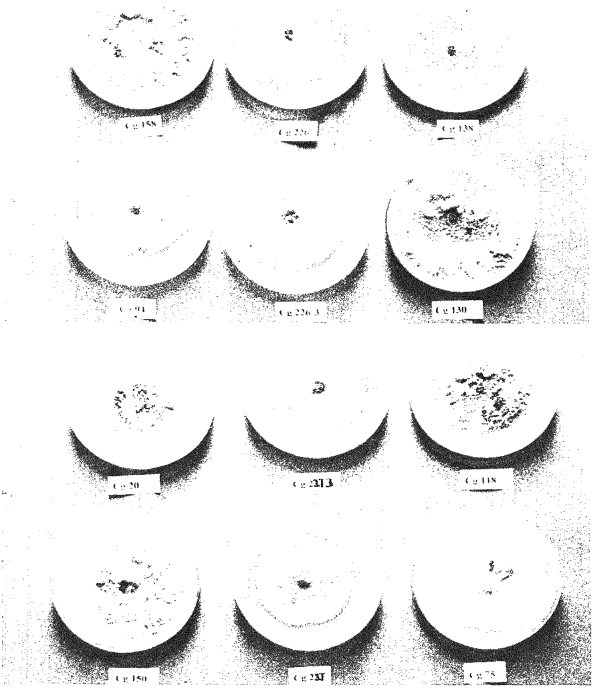

Cg 158
Cg 226
Cg 138
Cg 91
Cg 226.3
Cg 130
Cg 20
Cg 213
Cg 118
Cg 150
Cg 237
Cg 78

Plate 23

Plate 24: Three sorghum lines least affected by any of the 12 isolates of *C. graminicola*

IS 3089 - Infected by Cg 227-3
IS 3758 - Infected by Cg 118
IRAT 204 - Not infected

Plate 24

Plate 25: Three sorghum lines least affected by any of the 12 isolates of *C. graminicola*

A 2267-2 - Not infected
IS 18442 - Infected by Cg 150
IS 8354 - Infected by Cg 130

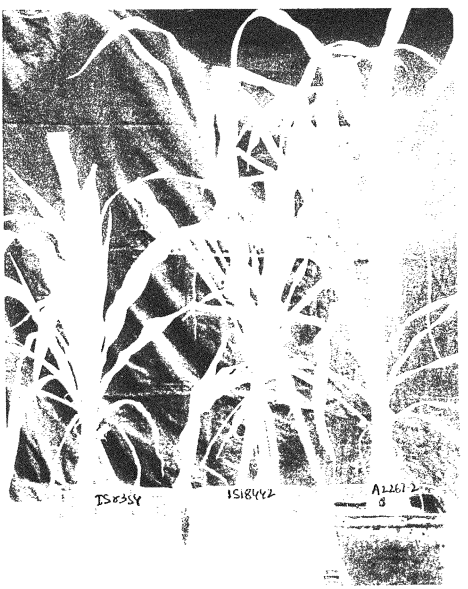


Plate 25

Plate 26: Variation in disease severity (aggressiveness) caused by Cg 138 and Cg 118 on sorghum line IS 3089

Plate 26

Plate 27: Random amplified polymorphic DNA (RAPD) analysis of 12 isolates of *C. graminicola*
(1-Cg 20; 2-Cg 75; 3-Cg 94; 4-Cg 118;
5-Cg 130; 6-Cg 138; 7-Cg 150; 8-Cg 158;
9-Cg 226; 10-Cg 226-3; 11-Cg 227; 12-Cg 227-3;
M-Marker)

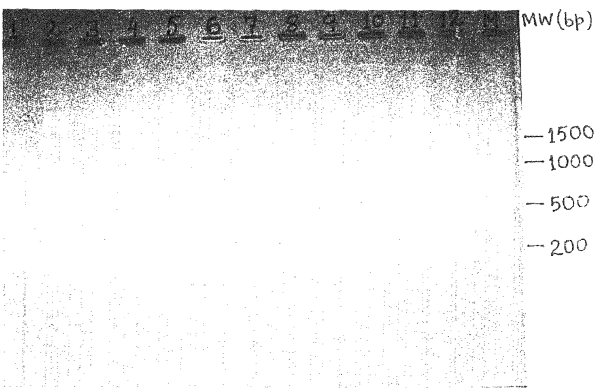


Plate 27

DISCUSSION

CHAPTER V

DISCUSSION

Anthracnose is the most destructive disease of sorghum in warm, humid region, but the pathogen *Colletotrichum graminicola* is cosmopolitan in distribution in varied climatic conditions. Although *C. graminicola* has a wide host range among cereals and grasses, isolates from one host do not necessarily infect other hosts. The capability of an isolate to infect more than one host: is not a general agreement (Ali and Warren, 1992). However, variability in biology, pathology and molecular level among isolates of *C. graminicola* from sorghum is reported by several workers which pose a threat to resistance breeding against the disease.

In present investigation, significant differences were observed with respect to shape and size of the conidia of *C. graminicola* among 12 isolates. Similar observations were made by Tiffany and Gilman (1954) and Pande *et al.* (1991), though Pande and coworkers did not observe significant differences in the breadth of the conidia.

The size of the conidia of monoconidial isolate of Cg 226-3 was different from its parent isolate which supports, intrapopulation variability in morphological characters of the pathogen reported by Mathur *et al.* (1997). However, in another monoconidial isolate Cg 227-3, the conidial size was not significantly different from its parent isolate Cg 227.

Conidial dimorphism in some isolates was an usual phenomenon of the fungus and unicellular conidia of the pathogen have been described

as falcate, lunate or spindle shaped (Mordue, 1967; Sutton, 1980). In addition to falcate conidia smaller oval shaped conidia were produced by this fungus but this differences in conidial morphology has been attributed to irregular form of falcate conidia (Mordue, 1967). The only published account of oval conidia from *C. graminicola* was by Nishihara (1975) who described them as distinct from falcate conidia and concluded that oval conidia were more virulent than falcate conidia on corn. But it still could not discount the possibility that oval conidia might be irregular or abortive form of falcate conidia through abnormal development, although ontogenical distinction was confirmed (Panaccione *et al.*, 1989). The present studies also showed that most of the isolates produced spindle shaped conidia besides the falcate conidia. The ontogenical and developmental relationship between falcate and spindle shaped conidia may need further investigation.

Among the 12 isolates, two isolates (Cg 94 and Cg 150) failed to produce appressoria. This probably relates to Dey's (1919) observation that agar surface do not always provide sufficient stimulation for differentiation of appressoria in *Colletotrichum*. Appressoria formation is primarily controlled by the fungal genotype (Emmett and Parbery, 1975) but the expression of this genotype appears to be controlled by growth surface of the germ tubes (Lapp and Skoropad, 1978; Van Dyki and Mims, 1991). Therefore, virulence of these two isolates could not be correlated with the production of appressoria on the culture medium.

Wide variation among isolates was observed in size of the appressoria which is in confirmation with Mordue (1967). However, majority of

appressoria were within the range given by Sutton (1980) in case of *C. sublineolum* (=*C. graminicola* from sorghum) but in isolates Cg 20, Cg 130 and Cg 158 the appressoria size was similar to that of *C. graminicola* (Sutton, 1980).

Appressoria of all isolates were terminal but in isolate Cg 130 intercalary appressoria were also found which is a rare character, sometimes observed in *C. graminicola* (Sutton, 1980). Although Sutton (1968) described appressoria of *C. graminicola* as aseptate, it was observed probably for the first time the septation in intercalary (in Cg 130) as well as in terminal (in Cg 20) appressoria with a rare frequency. However, formation of 1-2 septa in appressorium is a common character of several other species of *Colletotrichum* where both cells function as individual appressoria (Sutton, 1962).

Shape of the appressoria is an important character of taxonomic value. Von Arx (1957) used morphology of appressoria from germinating conidia of *C. crassipes* to key out this species from the other straight spore species. Sutton (1962) suggested the possibility that appressoria character may be used more extensively in identification of *Colletotrichum* species. In the present study all the isolates produced appressoria which were obovate, elliptical, pyriform to broadly clavate in shape, but only isolate Cg 138 produced appressoria with irregular shape, forming some lobes. Sutton (1968; 1980) mentioned the production of appressoria with lobes in *C. graminicola* from sorghum.

Variation in number and size of setae were wide among the isolates. In 10-day-old culture all isolates except Cg 118 produced setae. Though production of setae is a genetically controlled character (Sutton and Waterson, 1970), atmospheric humidity plays an important role for expression of this genetic potential (Frost, 1964). Development of setae is also related to the type of primordium for acervulus initiation (Maiello, 1988). Setae are generally not associated with acervuli derived from hollow ringed primordia. Acervuli originating from mycelial knots produce abundant setae. Variation in number of setae among 12 isolates may be due to the types of primordium formed for acervulus initiation as cultural conditions were same for all isolates. Isolates Cg 118 produced setae sparsely after 20 days of incubation which indicated that it is capable of producing setae under certain conditions and time. Even though all isolates were maintained under the same conditions it failed to produce setae in three repreated experiment. This could, therefore, be attributed to its morphological diversity.

Production of setae could not be correlated with the virulence of the isolate. Isolate Cg 20 and Cg 118 were in the same group according to virulence index with maximum and minimum setae, respectively.

One isolate Cg 150 produced two types of setae. A second type of setae which was bearing conidia on its apex was similar as described by Lenne *et al.* (1984). Production of conidia from setae in *Colletotrichum* was described by several workers (Ikata, 1936; Ling and Liu, 1944; and Mordue, 1967), but it was not mentioned by Sutton (1980, 1992). Lenne *et al.* (1984) claimed that it is a character of several species of *Colletotrichum*

including *C. graminicola* and was overlooked in previous studies. But in this study, among 12 isolates of *C. graminicola* from sorghum conidia-producing setae was found only in one isolate in OMA medium. It was found that setae with conidia and normal conidiophore emerged from the same point of the hyphae (Plate 19), and conidiophore also with septation and light melanization near the point of emergence similar to the setae. This indicated the possibility of similarity in morphogenesis of setae and conidiophore during primary stage of initiation. However, detailed study is required to confirm the ontogenic relationship of conidiophore and setae.

Branched setae was found only in Cg 227-3 which is also an uncommon character of *C. graminicola* but could not be correlated significantly with other variabilities of the pathogen other than morphology.

Two types of acervulus distinct and indistinct were produced in different isolates. Similar results were also reported by Rao *et al.* (1998), but unlike their observation, the number of setae and distinction of acervulus could not be correlated. Possibly, this was due to the types of primordia involved in acervulus initiation in these 12 isolates (Maiello, 1988).

Among the 12 isolates, 9 produced sclerotia within 20 days of incubation and 3 isolates (Cg 130, Cg 158 and Cg 227) failed to produce sclerotia. It was reported that formation of sclerotia fully depends upon the isolates of *Colletotrichum* (Sutton, 1980; Khan and Sinclair, 1992). Although there was no significance of sclerotia on virulence of the pathogen but it helps the fungus to survive for a long period in soil, plant debris and disseminate to the new crop.

Cultural characteristics on OMA medium was similar to those described by several workers (Chowdhury, 1936; Mordue, 1967; Sutton, 1980; Jamil *et al.*, 1989; Saifulla *et al.*, 1990; Pande *et al.*, 1991). However, these descriptions varied according to media and incubation temperatures used for the study. Cultural characteristics of 12 isolates varied in respect to form, colour, size of the colony and sporulation. Four of these 12 isolates, Cg 94, Cg 118, Cg 130 and Cg 138 produced form and colour of the colony as described by Sutton (1980) for *C. graminicola* (from maize) and other 8 isolates produced colony similar to *C. sublineolum* (= *C. graminicola* from sorghum). But all the colony characters of 12 isolates were similar to those described by Mordue (1967) and Pande *et al.* (1991).

Significant differences were recorded in radial growth and sporulation among 12 isolates. Similar variations were also reported by earlier workers (Mishra *et al.*, 1980; Jamil *et al.*, 1989; Saifulla *et al.*, 1990; and Pande *et al.*, 1991) using isolates of *C. graminicola* from different hosts as well as the same host. Growth and sporulation of the pathogen fully depends upon media, temperature, light exposure and humidity maintained in the study. Good sporulation ($>10^5$ conidia/mm^2) was recorded in Cg 118, Cg 226, Cg 227 and Cg 227-3, but poor sporulation occurred in other isolates. Although good sporulation has been reported in continuous light at 24°C (Mishra and Siradhana, 1980; Jamil *et al.*, 1989) on OMA medium (Chowdhury, 1936; Jamil *et al.*, 1989), Saifulla and Ranganathaiah (1990) reported poor sporulation on OMA medium at 25°C. So, it could be concluded that in this study sporulation varied widely depending upon the isolates and this is similar to those reported by Mishra

and Siradhana (1980). Two monoconidial isolates were not significantly different from their parent isolates for colony growth but were different in respect to sporulation.

According to the disease reaction isolates could be differentiated into three pathogenic races. Earlier presence of races of *C. graminicola* in different states (Pande *et al.*, 1991) as well as within a state in India (Rao *et al.*, 1998) have been reported which supports this result. Resistance to anthracnose is governed by single dominant gene (LeBeau *et al.*, 1950; Frederiksen and Rosenow, 1971) showing single locus segregration with multiallelism (Murty *et al.*, 1990). It has also been reported that the allele responsible for resistance in one host genotype against one pathotype may not provide resistance against the other pathotypes (Tenkouano and Miller, 1993). However, there is no information on the number of resistance loci as well as the genes showing partial or moderate resistance or delayed disease development.

Virulence index was calculated to determine pathogenic potential of an isolate using three independent pathogenicity parameters latent period, virulence and aggressiveness. Isolates were significantly different for virulence index. The grain isolate Cg 226 and its monoconidial derivative Cg 226-3 not only caused leaf infection but also produced highest virulence index as compared with other isolates. Although resistance to kernel and leaf infection is independent to each other, interaction between resistance genes is unknown (Warren and Shepherd, 1976). In case of monoconidial isolates virulence index was not significantly different.

Morphological and cultural variability could not be well correlated with pathogenic variability as these traits are governed by different genes. Similar results were reported by Thomas *et al.* (1995).

Isolates were divided into four groups according to the cluster analysis based on DNA polymorphism from RAPD analysis. Molecular analysis showed more diversity than virulence analysis using host differentials. This finding is well supported by the earlier reports of Casela *et al.*, 1993). This is quite expected because gene(s) controlling a particular character most likely present a minute fraction of cellular DNA (nuclear and mitochondrial), whereas RAPD banding pattern obtained from total DNA reflect total DNA divergence (Andebrhan *et al.*, 1994). Relatively higher genetic heterogeneity of Indian populations of *C. graminicola* has also been observed through DNA analysis (Guthrie *et al.*, 1992). In the present study the polymorphism could not be correlated with the geographical origin of the isolates. Similar observations have been reported by Ouellet *et al.* (1993) in case of *Fusarium graminearum* and Casela *et al.* (1992, 1995) in *C. graminicola*, though in some cases importance of geographical regions were correlated (Guthrie *et al.*, 1992).

Among two monoconidial isolates Cg 226-3 was closely related to its parent isolate Cg 226 in its number and pattern of bands, but Cg 227-3 showed wide polymorphism with parent isolate Cg 227. Polymorphism among monospore cultures from the same basidiocarp was detected in *Crinipellis perniciosa* isolates and the reason was attributed to the heterothallism of the fungus (Andebrhan *et al.*, 1994). Under natural condition sexuality in *C. graminicola* has not been discovered so far,

therefore parasexual DNA exchange may be responsible for genetic variability as has been described in case of *Magnaporthe grisea* (Zeigler *et al.*, 1997). Intrapopulation variability at molecular level also was observed in *C. graminicola* and cluster composition of RAPD and virulence index were independent to each other (Mathur *et al.*, 1998).

Poor reproducibility of RAPD reaction has been reported by several workers (Guthrie, 1993). In this study the reaction was run thrice and the results were consistent showing high reproducibility of the method used. This is supported by several other reports for a number of plant pathogens (Manulis *et al.*, 1994; Grajal-Martin *et al.*, 1993; Abadi *et al.*, 1996) with a little fluctuation in intensity and number of bands. However, consistency in reproducibility depends upon the primer and its sensitivity to variation in assay conditions, such as concentrations of $MgCl_2$ or the quality of DNA (Ouellet *et al.*, 1993).

All these four types of studies to determine the variability among 12 isolates were tried to correlate but could not be correlated exactly. But from overall result of this experiment it was found that Cg 118, Cg 138, Cg 226 (with its monoconidial isolate Cg 226-3) and Cg 227 were most diverse isolates than others. Out of these four isolates Cg 118, Cg 226 and Cg 227 produced either distinct symptom or infected different plant parts in their native host plants. But producing very similar symptoms like other leaf isolates Cg 138 showed greater diversity in all respects.

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SUMMARY AND CONCLUSIONS

CHAPTER VI

SUMMARY AND CONCLUSIONS

Anthracnose of sorghum (*Sorghum bicolor* (L.) Moench) caused by *Colletotrichum graminicola* (Ces.) Wilson (=*Colletotrichum sublineolum* Henn. Kabat *et* Bub.) is an economically important disease throughout the world, especially in warm and humid climate. The pathogen is highly variable and a number of pathotypes/races are known to exist. In this investigation pathogenic variability was studied among 12 isolates of *C. graminicola* using a set of six host differential lines. The isolates were collected from four major sorghum growing states (Maharashtra, Karnataka, Andhra Pradesh and Tamil Nadu) of India. These included 8 leaf isolates (Cg 20, Cg 75, Cg 94, Cg 118, Cg 130, Cg 138, Cg 150, Cg 158), 1 grain isolate (Cg 226), 1 mid rib isolate (Cg 227) and 2 monoconidial isolates (Cg 226-3 and Cg 227-3) derived from grain and midrib isolates. The isolates were also characterized for morphological, cultural and molecular diversity.

All the isolates were grown on OMA medium in petriplates and incubated at 25±2°C for morphological and cultural studies.

Microscopic studies revealed that conidia of all isolates varied widely in respect to their size and shape. Isolate Cg 227 had the largest (27.29 µm) conidia and Cg 138 had the smallest (22.27 µm) conidia. Based on size of conidia the isolates could be divided into 6 distinctly separate groups. Among 12 isolates 3 (Cg 94, Cg 227, Cg 227-3) produced only falcate conidia and others showed conidial dimorphism producing both falcate and spindle shaped conidia.

Significant variation in shape and size of appressoria was found among isolates. Largest appressoria was produced by Cg 20 and Cg 130 in length and breadth respectively whereas Cg 226 and Cg 226-3 produced smallest. Lobed appressoria was found only in isolate Cg 138. Septation of appressoria was observed for the first time in case of Cg 20 and Cg 130.

All isolates produced setae varying in number per acervulus and size except Cg 118 which was unable to produce setae. Isolate Cg 150 produced a second type of setae having a conidia at the apex.

Isolates Cg 130, Cg 158 and Cg 226 failed to produce sclerotia even 20 days after incubation. Whereas rest of the isolates were able to produce sclerotia within 20 days.

Cultural characteristics of the pathogen varied widely regarding colony growth and sporulation. Isolate Cg 226, Cg 227 and Cg 227-3 showed good sporulation (>10^5 conidia/mm^2 of the colony) but colony growth was less when compared to other isolates. Growth pattern and colony colour also differed to a great extent.

For pathogenicity test six sorghum lines (A 2267-2, IRAT 204, IS 8354, IS 3758, IS 3089, IS 18442) were used as host differentials. Pot-grown 21 day-old seedlings were inoculated by each isolate (10^5 conidia/ml) in a greenhouse. Data on disease reaction and disease severity were recorded 14 days after inoculation, while for latent period 2 days onwards after inoculation.

In pathogenicity studies, two sorghum lines A 2267-2 and IRAT 204 showed resistant reaction whereas other two lines IS 3089 and IS 18442 gave susceptible reaction to all isolates. Isolates produced mixed reaction towards IS 8354 and IS 3758. According to disease reaction isolates could be divided into 3 pathotypes - Cg 118 and Cg 138 in one group; Cg 158, Cg 226, Cg 226-3 and Cg 227 in another group; and rest of the 12 isolates were in last group. Interestingly monoconidial isolate Cg 227-3 gave moderately resistant reaction to IS 3758 unlike to its parent isolate Cg 227.

Wide variation was observed regarding latent period. Minimum latent period observed in case of Cg 226 towards IS 3758 and maximum for Cg 118 towards IS 3089 considering susceptible lines.

Aggressiveness was highest in case of Cg 226 and lowest in Cg 118. Monoconidial isolate Cg 226-3 was much less aggressive on an average than the parent isolate Cg 226.

Considering 3 parameters (latent period, virulence and aggressiveness) virulence index was calculated for each isolate indicating the potential of an isolate to damage the crop. Virulence index was highest in Cg 226 and lowest in Cg 130.

Dendrogram based on aggressiveness and virulence index divided the isolates into 3 and 4 cluster respectively while Cg 226 showed maximum diversity compared to other isolates. Isolate Cg 227 and its monoconidial isolate Cg 227-3 was in same cluster in both cases.

Random Amplified Polymorphic DNA (RAPD) analysis indicated a wide diversity among isolates at molecular level using Primer OPA8 (base sequence 5'GTGACGTAGG3'). It was observed that banding pattern of Cg 138 varied greatly than others. Molecular weight of the bands ranged around 0.2 Kb to 2.0 Kb. Based on dendrogram isolates could be grouped into 4 clusters with isolate Cg 138 alone in one cluster; Cg 226, Cg 226-3 and Cg 227 in second cluster, Cg 158, Cg 227-3 and Cg 20 in third cluster; and rest of the isolates i.e. Cg 118, Cg 94, Cg 75, Cg 130, Cg 150 were in fourth cluster. Surprisingly isolate Cg 227 and its monoconidial isolate Cg 227-3 were included into separate cluster.

It was evident from this investigation that Indian populations of *C. graminicola* is highly variable in respect to their morphological, cultural, pathogenic and molecular characters and 3 distinct pathotypes were identified from 12 isolates. But no significance of their geographical distribution was found. Intrapopulation variability in grain and midrib isolates were confirmed by all four types of analysis. Besides, it was also proved that grain and midrib isolates are equally or sometimes more efficient compared to leaf isolates to cause disease on leaf. Expectedly molecular analysis using RAPD markers showed greater diversity among isolates than virulence analysis. Although it was difficult to correlate 4 different variability studies but Cg 118, Cg 138, Cg 226 (with its monoconidial isolate Cg 226-3) and Cg 227 showed greater diversity in all studies.

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Original not seen

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APPENDIX

Description of sorghum lines used for pathogenic variability study

Designation	Race	Origin
A 2267-2	Caudatum	India
IRAT 204	Durra caudatum	Burkina Faso (Africa)
IS 8354	Caudatum bicolor	USA
IS 3758	Caudatum bicolor	USA
IS 3089	Caudatum bicolor	USA
IS 18442	Guinea durra	India

Source: Thakur (1995); Thakur *et al.* (1998)