STUDIES ON THE MECHANISMS OF RESISTANCE IN PEARL MILLET GENOTYPES TO DOWNY MILDEW DISEASE

BY

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M. Sc.(Ag)

THESIS SUBMITTED TO THE
ACHARYA N.G. RANGA AGRICULTURAL UNIVERSITY
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE AWARD OF THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN THE FACULTY OF AGRICULTURE

DEPARTMENT OF PLANT PATHOLOGY
COLLEGE OF AGRICULTURE
ACHARYA N.G. RANGA AGRICULTURAL UNIVERSITY
RAJENDRANAGAR, HYDERABAD - 500 030

SEPTEMBER, 1996

CERTIFICATE

Ms. V. Celia Chalam has satisfactorily prosecuted the course of research and that the thesis entitled **STUDIES ON THE MECHANISMS OF RESISTANCE IN PEARL MILLET GENOTYPES TO DOWNY MILDEW DISEASE** 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 her for a degree of any University.

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This is to certify that the thesis entitled **"Studies on the mechanisms of resistance in pearl millet genotypes to downy mildew disease"** submitted in partial fulfillment of the requirements for the degree of **'DOCTOR OF PHILOSOPHY IN AGRICULTURE'** of the Acharya N.G. Ranga Agricultural University, Hyderabad, is a record of the bonafide research work carried out by **Ms. V. Celia Chalam,** 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. The published part has been fully acknowledged. All assistance and help received during the course of the investigations have been duly acknowledged by the author of the thesis.

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CONTENTS

LIST OF ILLUSTRATIONS

LIST OF PLATES

LIST OF TABLES

Contd...

ACKNOWLEDGEMENTS

I express my deep sense of gratitude and sincere thanks to Dr. K. Chandrasekhara Rao, Chairman of the advisory committee, Professor and Head, Department of Plant Pathology, College of Agriculture, Rajendranagar, Hyderabad for his encouragement, valuable suggestions and guidance during the course of this study.

I specially thank Dr. S.D. Singh, Senior Scientist, Cereals Pathology, Crop Protection Division, (CPD), ICRISAT Asia Center for his keen interest, constant supervision, constructive criticism and invaluable suggestions throughout my work at ICRISAT.

I am highly thankful to other members of the Advisory Committee Dr. M.S.S. Reddy, Professor and Head, Department of Genetics and Plant Breeding, Acharya N.G. Ranga Agricultural University and Dr. K.C. Mohankumar, Associate Professor, Department of Agricultural Microbiology and Bioenergy, Acharya N.G. Ranga Agricultural University, for their help during the course of this study.

I take this opportunity to express my gratitude to Dr. V. Subramanian, Senior Scientist, Cellular and Molecular Biology Division (CMBD), ICRISAT, for helping me in carrying out the biochemical aspects during the course of this study.

Thanks are also due to all the staff of the Department of Plant Pathology, Acharya N.G. Ranga Agricultural University for their help and cooperation during the study period.

Special thanks to Mr. P.J. Mohan Rao, Cereals Pathology, CPD, ICRISAT, for the continuous help in carrying out the experiments. I sincerely thank all the staff of the Cereals Pathology, CPD, for their helping hand throughout my research at ICRISAT. I sincerely thank Mr. P. Suryaprakash of CMBD, ICRISAT, for his help during biochemical analysis.

I express my sincere thanks to Mr. A.K. Murthy, Senior Engineer, Electron Microscope Lab., CPD, ICRISAT, for helping me in the Microtomy work. I am highly thankful to Mr. V.R. Prabhakar, Mr. Nageswar Reddy and Mrs. P. Padmavathy, ICRISAT, for their help in statistical analysis and graphical illustrations. My sincere thanks are also due to Mr. D. C. Thyagaraj, Repographic Unit, and to all the staff of Library and Documentation Services, ICRISAT for their timely help.

I am thankful to Dr. D.L. Oswalt and Dr. B. Diwakar, Training and Fellowship Program, ICRISAT, for permitting me to work at ICRISAT Asia Center.

To my beloved late father whose blessings from heavenly abode are always a source of wisdom and inspiration for me and the affectionate and blissful encouragement of my mother is respectfully acknowledged. I extend my sincere thanks to my brothers and sisters for their constant encouragement throughout my academic career.

It is my pleasure to acknowledge my friends Vijaya, Prasad and Prasanna who stood by my side throughout my research and made my work a pleasant experience.

I sincerely thank Acharya N.G. Ranga Agricultural University and Council of Scientific and Industrial Research (CSIR) for providing financial assistance during the course of the study.

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DECLARATION

I, **V. Celia Chalam,** hereby declare that the thesis entitled "**STUDIES ON THE MECHANISMS OF RESISTANCE IN PEARL MILLET GENOTYPES TO DOWNY MILDEW DISEASE**" submitted to ACHARYA N.G. RANGA AGRICULTURAL UNIVERSITY for the degree of **Doctor of Philosophy in Agriculture** is a bonafide record of work done by me during the period of research at the Asia Center of the International Crops Research Institute for the Semi-Arid Tropics (IAC), Patancheru, A.P., 502 324, India. This thesis has not formed in whole or in part, the basis for the award of any degree or diploma.

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Degree to which it is submitted : DOCTOR OF PHILOSOPHY

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Year of submission : 1996

Abstract

Studies on histopathological, anatomical, and biochemical differences between resistant and susceptible pearl millet genotypes in relation to downy mildew disease were undertaken at the ICRISAT Asia center, Hyderabad. Indirect sporangial germination by producing zoospores was observed. Zoospore germ tubes penetrated the leaves directly through the epidermal cells, with or without appressoria and very rarely through stomata. The pathogen colonized the leaf tissue inter-and intracellularly.

Germination of sporangia and zoospores, and penetration was significantly higher in susceptible genotypes than the resistant ones. Sporangial and zoospore germination, and penetration decreased with the increase in leaf age. The sporangial and zoospore germination and penetration were highest at 20°C and decreased with the further increase or decrease in temperature. In resistant genotypes, 0-20 per cent leaf samples were colonized by *S. graminicola*, where as in susceptible ones, colonization was 100 per cent. Colonization was extremely restricted in resistant genotypes.

With few exceptions, cuticle thickness was significantly more in resistant genotypes than the susceptible ones. The wax content was significantly more in the three resistant genotypes (IP 18293, IP 18294, and IP 18296), than the susceptible ones with the exception of IP 18292 (resistant), in which wax content was less than the other resistant and susceptible genotypes. A linear increase in the cuticle thickness and wax content with the increase in leaf age was observed. Significant differences in number of stomata were not observed between the susceptible (HB 3 and 7042 S) and resistant (IP 18294 and IP 18296) genotypes on both the surfaces. Similar observations were also made between resistant genotype IP 18292 and susceptible genotype HB 3 on the upper surface.

Total phenols were significantly more in resistant genotypes than the susceptible ones, both in inoculated and non-inoculated leaves. There was an increase in total phenols in both susceptible and resistant genotypes after inoculation. In case of resistant genotypes, IP 18292 and IP 18293, a distinct band was identified close to the solvent front which is absent in susceptible ones, HB 3 & 7042 S. Total phenols and total soluble sugars (TSS) increased with the increasing age of the seedlings. Total soluble sugars did not differ significantly between inoculated and non-inoculated leaves in resistant genotypes, while in susceptible genotypes, the TSS were more in inoculated leaves.

INTRODUCTION

CHAPTER I

INTRODUCTION

Pearl millet (*Pennisetum glaucum* (L.) R. Br.) is an important food crop in the semi-arid tropics of the Indian subcontinent and many African countries. The total area under pearl millet cultivation is about 26 million hectares (Singh, 1995). The crop is grown on the poorest soils and under harsh climatic conditions where no other crop can probably be grown. Although the crop is quite hardy, it still suffers from various abiotic and biotic stresses. One of the major biotic yield-reducing factors is the disease downy mildew, caused by *Sclerospora graminicola* (Sacc.) Schröt. The disease is of great economic importance in India and in many parts of Africa.

The downy mildew pathogen was first reported on pearl millet in India in 1907 (Butler, 1907). Initially, the disease was not considered serious but the true magnitude of the losses has now been fully appreciated. In 1971, the disease appeared in an epidemic form in the Indian sub-continent resulting in the withdrawal of the most popular hybrid HB 3 which had contributed to a record harvest of 8 million tones in 1970-71 (AICMIP, 1972). Subsequent to this epidemic, grain yield losses continued to occur quite frequently due to downy mildew epidemics in India (Singh *et al.*, 1987).

Crop protection from the diseases is one of the mandates given by humanity to plant pathologists. Exploitation of host plant resistance is the watchword of every agriculturist in the developed and developing countries. A thorough understanding of the mechanism(s) of resistance operating in pearl millet genotypes to downy mildew is highly essential to develop varieties with stable and durable resistance. The role of sporangia in the epidemiology of pearl millet downy mildew was clearly established (Singh and Williams, 1980). Subramanya *et al.* (1983) studied the biology of systemic infection of pearl millet downy mildew by zoospores. The response of plants and callus cultures of resistant and susceptible cultivars of pearl millet and sorghum to inoculation with asexual spores of *S. graminicola* and *Peronosclerospora sorghi* was studied (Mauch-Mani *et al.*, 1989; Shetty, 1989). Murty (1980) suggested that for quick screening against downy mildew, assaying resistance in terms of biochemical genetic parameters which are less influenced by the environment would be more reliable. The phenolic content and activities of peroxidase and polyphenol oxidase were found to be related to degree of resistance (Thukral *et al.*, 1986). However, information on quantitative histopathological aspects and anatomical and biochemical mechanisms operating in pearl millet against *S. graminicola* is inadequate. Therefore, the present study was undertaken to determine the:

- germination of sporangia and zoospores on different parts of the second leaf of a resistant and a susceptible genotype.

- germination of sporangia and zoospores on the middle parts of the second leaf of resistant and susceptible genotypes.

- germination of sporangia and zoospores on 1st to 8th leaf of a resistant and a susceptible genotype.

- penetration of the second leaf of resistant and susceptible genotypes by zoospore germ tubes.

- penetration of 1st to 8th leaf of a susceptible and a resistant genotype by zoospore germ tubes.

- effect of temperature on *in vivo* germination of sporangia and zoospores, and leaf penetration by germ tubes.

- colonization of 1st to 5th leaf by *S. graminicola* in resistant and susceptible genotypes.

- association of resistance/susceptibility with several anatomical factors including:
  - (i) Thickness of cuticle.
  - (ii) Epicuticular waxes.
  - (iii) Stomata.

biochemical basis of resistance/susceptibility involving:

(i) Total phenols.

(ii) Total soluble sugars.

REVIEW OF LITERATURE

CHAPTER II

REVIEW OF LITERATURE

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

1) General
2) Histopathology
3) Infection in older tissues by the downy mildew pathogens.
4) Penetration and colonization by the downy mildew pathogens in resistant varieties.
5) Effect of environmental factors on sporangial germination and infection by the downy mildew pathogens.
6) Mechanisms of disease resistance in plants.

2.1 General

2.1.1 Historical

The downy mildews constitute an important group of plant diseases. They are so called because of the downy or wooly growth produced by the pathogens

on the infected leaf areas. Downy mildew pathogens, belonging to the family Peronosporaceae, are obligate parasites, although a few of them have been grown in tissue culture (Arya and Tiwari, 1969; Tiwari and Arya, 1969; Safeeulla, 1970, 1975; Bhat, 1973; Bhat *et al.*, 1980).

Among the diseases affecting pearl millet (*Pennisetum glaucum* (L.) R. Br.), downy mildew (*Sclerospora graminicola* (Sacc.) Schröt.), also known as 'green ear' disease is the most devastating one. *S. graminicola* is the type species of the genus. It was originally named as *Protomyces graminicola* by Saccardo in 1876 who reported it on *Setaria verticillata*. Subsequently, Schröter in 1879 renamed it as *S. graminicola* in his work on the genus *Sclerospora* in Germany (Ullstrup, 1973). In 1884, Farlow described *S. graminicola* on *S. viridis* (L.) Beauv. In India, the earliest investigations on pearl millet downy mildew were carried out by Butler (1907) and Kulkarni (1913). The disease was not considered serious, since damage was severe only where pearl millet was grown in low-lying, poorly-drained areas (Butler, 1918). Mitter and Tandon (1930) reported the seriousness of the disease in low-lying areas in the vicinity of Allahabad in North India. With the release of high yielding hybrids (HB 1, HB 2 and HB 3) in certain parts of India, the disease became serious resulting in an epidemic during 1971-72 (AICMIP,1972). Downy mildew is considered a major biotic yield reducing factor in pearl millet not only in India, but also in many countries in Africa (Singh, 1995).

2.1.2 Geographical distribution

S. graminicola is widely distributed in the tropical areas of the world, and is especially important in India (Nene and Singh, 1976) and Africa (N'Doye *et al.*, 1986; Chevaugeon, 1952; Saccas, 1954; Bouriquet, 1963). In India, the pathogen is present in all the states where pearl millet is cultivated. However, the disease was not reported on pearl millet in the American countries (Singh *et al.*, 1993).

2.1.3 Economic importance

Initially, the disease was not considered serious but the true magnitude of the losses has now been fully appreciated. Reports suggest 6 per cent loss in east China (Porter, 1926), 45 per cent in Allahabad, India (Mitter and Tandon, 1930), and up to 27 per cent loss from 1962 to 1964 in Rajasthan state, India (Mathur and Dalela, 1971). According to reports from Africa, there was 60 per cent loss in Mozambique (DeCarvalho, 1949), 10 per cent in Nigeria (King and Webster, 1970) and 0-50 per cent in other western African Countries (CILSS 1986; CILSS 1987; Selvaraj, 1979; Saccas, 1954). Although comprehensive data for grain yield loss in pearl millet due to this pathogen is not available (Singh *et al.*, 1993), the actual yield reducing potential of the disease has been fully recognized. This was clearly demonstrated in HB 3, a popular hybrid in India in the early seventies, when pearl millet grain production was reduced from 8.2 million t in 1970-71 to 5.3 million t in 1971-72 (AICMIP, 1972). This reduction was, to a large extent, due to the downy mildew epidemic, in which yields in some fields were reduced by 60-70 per cent.

Subsequent to this epidemic, grain yield losses continued to occur quite frequently due to downy mildew epidemics in India (Singh *et al.*, 1987). It has been demonstrated that the losses in grain yield can be directly proportional to the disease severity (Williams and Singh, 1981).

2.1.4 Symptoms

There is considerable variation in the symptoms which almost always develop as a result of systemic infection. Systemic symptoms generally appear on the second leaf, and once these appear, all the subsequent leaves and panicles also develop symptoms, except in case of recovery resistance where plants outgrow the disease (Singh and King, 1988). The disease can appear on the first leaf also, under conditions of severe disease development. Leaf symptoms begin as chlorosis (yellowing) at the base of the leaf lamina, and successively higher leaves show a progression of greater coverage of leaf area by symptoms. The 'half- leaf' symptom, characterized by a distinct margin between the diseased (basal portion) and non-diseased areas towards the tip, occurs in pearl millet genotypes. It is similar to the half-leaf symptoms caused by *Peronosclerospora sorghi* on sorghum and maize. Under conditions of high humidity and moderate temperature, the infected chlorotic leaf areas support a massive amount of asexual sporulation, generally on the abaxial surface of leaves, giving them a downy appearance. Severely infected plants are generally stunted and do not produce panicles.

The name 'green ear' stems from the appearance of green panicles due to the transformation of floral parts into leafy structures, which can be total or partial. This is sometimes referred to as virescence. These leafy structures can also be chlorotic, and sometimes support sporulation. In certain cases, green ear is the only manifestation of the disease (Singh *et al.*, 1993; Singh, 1995).

Symptoms are rarely seen as local lesions or isolated spots on leaf blades (Saccas, 1954; Girard, 1975). Spots vary in shape and size, and are at first chlorotic, produce sporangia and later become necrotic.

2.1.5 Causal organism

S. graminicola produces two types of spores, asexual spores (sporangia, zoospores) and sexual spores (oospores). The whitish downy growth of the pathogen on the leaf surface is the "asexual phase". Oospores are produced within the infected leaf tissues (Singh *et al.*, 1993).

A. The asexual phase

This contains sporangiophores and sporangia. Sporangiophores are short, stout, determinate and dichotomously-branched structures that emerge from systemically infected leaves through stomata. Sporangia are produced on sterigmata located at the tips of sporangiophore branches. Fully developed sporangia are hyaline, thin-walled, ellipsoid or broadly elliptic and papillate, with

dimensions of 15-22 x 12-21 µm (Saccas, 1954; Jouan and Delassus, 1971). However, temperature can affect the size of sporangia (Singh *et al.*, 1987).

Sporangia germinate indirectly by producing zoospores. The number of zoospores per sporangium may vary from 1 to 12 (Shetty, 1987; Ramakrishnan, 1963). Zoospores emerge through a pore produced by the opening of the operculum in the apical region of the sporangium. Zoospores swim for 30-60 minutes (min), encyst, and then germinate by forming a germ tube. Sometimes, zoospores may germinate within the sporangium, in which case, the germ tube grows through the apical pore giving the appearance of direct germination (Shaw, 1981). However, direct germination, without the formation of zoospores, also occurs (Mauch-Mani *et al*, 1989; Singh, 1995).

B. The sexual phase

The process of sexual reproduction in *S. graminicola* is initiated with the production of antheridia (male) and oogonia (female) structures, which culminates in the formation of oospores. Oospores are produced in large numbers. The oospore wall has three distinct layers; the exosporium, the mesosporium, and the endosporium. As a rule the oogonial wall is fused with the oospore wall in *Sclerospora* spp., which is a major identifying feature of this genus. The mature oospore is spherical, brownish yellow, and measures 32 µm (22-35 µm) in diameter (Singh *et al.*, 1993).

Two mating types have been identified. These are considered necessary for the production of oospores, illustrating the heterothallic nature of the pathogen. However, there is also evidence of the existence of homothallism (Michelmore *et al.*, 1982).

Oospores are resting spores. The thick oospore-wall protects them from desiccation and probably serves as an impermeable membrane. Earlier reports suggest that oospores can survive from eight months to 10 years under laboratory conditions (Nene and Singh, 1976). Recently, oospores stored under laboratory condition for up to 13 years have shown viability, although germination was greatly reduced after two years of storage (Singh and Navi, in press).

Many workers have reported germination of oospores, both directly by germ tubes and indirectly by the liberation of zoospores, with great variation in the frequency of germination (Nene and Singh,1976). However, these results have generally not been reproducible (Singh *et al.*, 1993). Recently, Panchbhai *et al.* (1991) developed a repeatable method of oospore germination with sodium hypochlorite (Clorox).

2.2 Histopathology

In order to have a better understanding of the mechanism(s) of resistance, detailed understanding of the mode of penetration and development of the

pathogen inside the host is imperative. The literature available on this aspect in pearl millet downy mildew is reviewed under the following heads.

2.2.1 Pre-penetration and penetration

A. Coleoptile

In coleoptile, often a single zoospore was associated with a single infection site. The zoospores that encysted on the coleoptile, produced germ tubes within an hour (h) after inoculation. An appressorium formed at the tip of the germ tube, produced an infection peg which pierced the epidermal cell wall and swelled into a vesicle within 4 hours (h) after inoculation (Subramanya *et al.*, 1983). Mauch-Mani *et al.* (1989) reported three different penetration sites in coleoptile. Some germ tubes penetrated epidermal cells through the upper periclinal wall of the cell. The second place where penetration occurred was the stomata. The fungus penetrated the stomata without appressorium formation and formed vesicles in the substomatal cavity. The third penetration site was at the upper rim of the coleoptile. Only a few germ tubes could penetrate border cells directly. The pathogen formed vesicles inside the cells.

B. Leaf

Subramanya *et al.* (1983) reported that the zoospores penetrated the leaf directly as well as indirectly. In the direct penetration process, single zoospore

produced germ tube (12.6-21μ) and appressorium on the surface of the epidermal cells. An infection peg that arose from the tip of the appressorium penetrated the cell wall and enlarged into a primary vesicle within 24 h after inoculation. Soon, these primary vesicles developed secondary vesicles.

Natural openings in the form of stomata on the leaves serve as excellent sites for infection by zoospores. The germ tubes of the zoospores grow towards the stomata and enters the leaves through the stomatal slits.

However, Bhatnagar (1988) found that the per cent direct penetration between the junction of two epidermal cells by germ tubes of *S. graminicola* was highest in the leaves of different ages and the least penetration occurred through stomata. He reported that the germination, appressoria formation, and penetration was completed in 4 h after inoculation. Similarly, Mauch-Mani *et al.* (1989) reported that the germ tubes of *S. graminicola* were formed within 1.5 h after inoculation and by 3.5 h after inoculation, the fungus has penetrated the plant cells and begun to form vesicles. Shetty (1989) noted that the zoospores produced germ tubes within 30 min and the pathogen penetrated the plant cells within 3 h after inoculation.

Penetration of leaf at the borders of anticlinal epidermal cells was recorded by Mauch-Mani *eöt al.* (1989) and Shetty (1989). In most of the cases, single zoospores infected the cells but large groups of zoospores clumping together and penetrating the surface at one point or in row was also common (Shetty, 1989).

Mauch-Mani *et al.* (1989) noted that about one third of the germ tubes penetrated the plant tissue through stomata at the leaf base. Shetty (1989) also found that the penetration was more successful through the stomata at the base of the leaf. In general, direct penetration was observed only in the younger portion of the leaves still within the whorl of the plants. Penetration through stomata was observed in all the parts of the leaves (Subramanya *et al.*, 1983).

C. Hypocotyl

On the hypocotyl, zoospore germ tube penetrated the tissue, at the borders of anticlinal epidermal cell walls and formed vesicles in the cells (Mauch-Mani *et al.*, 1989; Shetty, 1989).

D. Roots

The zoospores of *S. graminicola* infect the roots either through the root hair or through epiblema cells at the zone of elongation of the roots. Penetration was not observed through the root cap. The number of infection sites declined in the zone of maturation. A single zoospore could infect the root epidermal cells. The zoospore swam for a while, adhered to the epidermal cell wall and encysted. Within an hour of inoculation, it germinated to produce a germ tube (8-28μ long) and an appressorium (3-5.5μ). The contents of the zoospore moved into the appressorium and a sharp infection peg arose from the appressorium which pierced the epidermal cell wall and swelled into vesicle (8-8.3 μ) within 4 h after

inoculation (Subramanya *et al.*, 1983). The maximum penetration was noticed when the roots were inoculated at the region of elongation (Shetty, 1989).

E. Flower

Production of mycelium in the ovaries after inoculation with sporangia at stigma has been reported (Singh, 1974). The process of infection of ovary by the zoospore germ tubes is not yet well understood, although some efforts in this direction were made by Ramesh and Safeeulla (1983).

2.2.2 Post-penetration

A. Coleoptile

After penetration has occurred in the coleoptile, secondary vesicles were formed from the primary vesicles within 24 h after inoculation. Thereafter, the fungus became intracellular. About 48 h after inoculation, the mycelial threads were found to originate from the tips of the secondary vesicle which developed into intercellular mycelium within 98 h after inoculation (Subramanya *et al.*, 1983).

B. Leaf

Singh (1974) observed hyphae of *S. graminicola* in all plant parts of infected plants viz., leaf sheath, midrib, leaf lamina, stem, mother axis, pedicel, roots and rootlets. In leaves, the hyphae mostly occur in the mesophyll cells and substomatal cavities. Sporangiophores coming out in groups through stomatal cavities were also seen. Mycelium was typically aseptate, branched, and coenocytic. Two types of haustoria viz., club shaped and long curved with branches were present.

After penetration, a stout primary vesicle (3-12μ) originated from the hyphal tip within the infected epidermal cell. The fungus which was so far intracellular, produced intercellular hyphae from the vesicles 48 h after inoculation. The hyphae first originated as thin thread from the tip of the secondary vesicles and after 96 h, they became intercellular. Symptoms were observed on the inoculated plants five days after inoculation (Subramanya *et al.*, 1983). Bhatnagar (1988) noted that, after penetration, *S. graminicola* moved into intercellular spaces of mesophyll cells and finally moved downward direction. Mauch-Mani *et al.* (1989) also recorded dense intercellular mycelium and by 24 h after inoculation the first forked haustoria had formed in mesophyll cells. Bhatia and Thakur (1991) reported that fungal mycelium was confined to intercellular spaces and mesophyll cavities of chlorophyllous cells of pearl millet leaves. Oospores were observed in mesophyll cells.

C. Hypocotyl

In the hypocotyl, vesicles were formed in the cells after penetration. In susceptible seedlings, hyphae emerged from these vesicles and the fungus colonized the tissue intercellularly. In resistant seedlings, either the fungus was surrounded by papillae, or the cells around the infection site became gradually necrotic and collapsed. In both the cases, no further fungal growth was noticed (Mauch-Mani *et al.*, 1989; Shetty, 1989).

D. Root

Singh and Pushpavathi (1965) observed the presence of intercellular hyphae in the stem, mother axis, floral buds, glumes, pistil and stamens but not in adventitious roots of pearl millet. However, Singh (1974) observed hyphae of *S. graminicola* in roots and rootlets.

Subramanya *et al.* (1983) reported that the roots act as excellent sites of infection. But Mauch-Mani *et al.* (1989) reported that the zoospore germ tubes entered root hair, but no further growth was registered.

E. Seed

Histopathological studies on seed from infected ears of field grown plants or from the apparently disease free plants showed the presence of mycelium in the embryos (Ahuja *et al.*, 1989).

2.3 Infection in older tissues by the downy mildew pathogens

It is a general phenomenon in downy mildews that they attack usually juvenile tissues, the younger the host plant, the greater the susceptibility to systemic colonization by these pathogens. Increase in resistance with increasing age has been observed in many downy mildew diseases by several workers. The literature available on this aspect is reviewed below.

Bhatnagar (1988) reported that the percentage of zoospore germination and germ tube penetration was greatly decreased with the increase in the age of pearl millet leaves. According to Subramanya *et al.* (1981), the main shoot of the plant was susceptible to downy mildew up to 26 days from emergence. However, the new tillers that emerges later are susceptible, but up to the same age. They concluded that the susceptibility of pearl millet crop throughout the growth phase is due to continued emergence of tillers. Singh and Gopinath (1985) found that highest incidence of downy mildew occurred in pearl millet in growth stage-I (i.e.$\leq$ 5 mm long) and incidence declined progressively when seedlings were inoculated after growth stage III ($\geq$10 mm long, first leaf still folded).

Mauch-Mani *et al.* (1989) reported that the age-dependant susceptibility is valid, not only for whole plant, but it can also be observed at the tissue level. The leaf base (youngest part of pearl millet leaf) is susceptible, whereas the physiologically older portions are resistant, even in cultivars considered to be susceptible. Similar conclusions were drawn by Shetty (1989).

Age-dependant or developmental-stage-dependant susceptibility and/or resistance reactions are also reported in other crops such as sorghum (Jones, 1978; Yeh and Frederiksen, 1980), maize (Rathore and Siradhana, 1988; Olanya *et al.*, 1993), sunflower (Patil *et al.*, 1992), tobacco (Cohen *et al.*, 1987), sugarbeet (Byford, 1981), cucurbits (Cohen, 1981), alfalfa (Stuteville, 1981), lettuce (Dickinson and Crute, 1974), and betelvine (Muller and Sleumer, 1934).

2.4 Penetration and colonization by the downy mildew pathogens in resistant varieties

Most of the plants have some mechanisms for either avoiding or reducing the potential impact of pathogens. In case of downy mildew pathogens, it appears that spore germination either by production of zoospore or directly by means of a germ tube is a nonspecific process. It occurs normally on any plant surface or inert material provided the physical environment is suitable (Crute and Dickinson, 1976; Viranyi and Henestra, 1976; Riggle, 1977; Subramanya *et al.*, 1983). The age of the host tissue or the presence of antagonistic microorganisms on host surface can influence the establishment of biotrophic relationship between potentially

compatible pathogens and their hosts. Therefore, only a proportion of attempted infection may result in the successful development of vegetative mycelia (Michelmore, 1981).

The sequential process of zoospore germination, penetration and infection apparatus formation were similar in susceptible and resistant pearl millet cultivars inoculated with *S. graminicola* (Mauch-Mani *et al.*, 1989; Shetty, 1989). The germ tube penetration was observed in both resistant and susceptible cultivar seedlings but soon papillae were formed and necrotic lesions developed preventing further development of the pathogen in resistant cultivars (Shetty, 1989).

Yeh and Frederiksen (1980) reported that sequence of stomatal penetration by *Peronosclerospora sorghi* is similar in both susceptible and resistant varieties of sorghum but subsequent mycelium development was distinctly different. Restriction of hyphal growth in resistant cultivars becomes apparent two days after inoculation. Similar results were obtained by Shabani (1978) in maize infected with *S. sorghi* (sorghum pathotype).

Similar and other types of downy mildew disease resistant reactions namely, necrosis in cruciferous plants (Wang, 1949) and soybean (Ersek *et al.*, 1982); hypersensitivity in alfalfa (Stuteville, 1981), lettuce (Maclean *et al.*, 1974) and sunflower (Mouzeyar *et al.*, 1993); differences in spore germination and differential colony growth in peas (Clark and Spencer-Phillips, 1994), lettuce (Viranyi and

Henestra, 1976; Lebeda and Reinink, 1991), grapevine (Dai *et al.*, 1992) and radish (Ohguchi and Asada, 1991) have been reported.

2.5 Effect of environmental factors on sporangial germination and infection by the downy mildew pathogens

Environment can affect the development and spread of plant diseases in different ways. It can affect the perpetuation or over wintering of the pathogens, the build-up of both primary and secondary inoculum, the dissemination of inoculum, germination, and host penetration. The environment can affect growth and development of the host prior to being infected in such a way as to affect its susceptibility. Finally, it can influence the actual development of disease after the plant has become infected.

There is well documented information on the environmental factors influencing germination of the sporangia of *S. graminicola.* Hirata and Takenouti (1932) reported sporangial germination through the release of zoospores when placed in water, providing adequate air, and temperatures between 12.5 to 29°C. The germination was not affected by light. According to Hiura (1935), the time required for sporangial germination was 30 min at 20°C, and 2.5-5 h at 6-7°C. The minimum, optimum, and maximum temperatures for the process were found to be 5-7°C, 19°C and 30-33°C, respectively. Wang (1936) reported that sporangia take about 40 to 50 min for germination. Safeeulla *et al.* (1963) more or less confirmed Hiura's work. Slightly more rapid germination was found in the dark than in light.

The time taken for the formation and liberation of zoospores varied between 35-180 min. Singh and Gopinath (1990) reported that sporangia germinated at 10-45°C. There was a gradual increase in germination with the increase in incubation time from 30 min to 2 h at temperatures from 10-35°C. Germination was most rapid at 40 and 45°C, but total germination at these temperatures did not increase more than 60 per cent even after 3 h. Optimum temperature for germination was 29.6°C and little germination occurred after 2 h 40 min. Germ tubes grew at 15-35°C and temperature has no influence on the germ tube length.

The infectivity of sporangia (zoospores), as judged by systemic infection of young seedlings, was greatly influenced by pre-inoculation temperature of the sporangial suspension and post inoculation temperature. Sporangia stored at 10 and 15°C for up to 12 h prior to inoculation produced >70 per cent downy mildew when used as inoculum. Similar levels of downy mildew also were recorded at 20, 25 and 30°C for up to 10, 7 and 4 h, respectively. Sporangial infectivity generally declined more rapidly at the higher incubation temperatures, with no infectivity after as little as 2 h incubation at the two highest temperatures, 40 and 45°C. Inoculated seedlings incubated at 30°C developed downy mildew symptoms as early as 3 days after inoculation. At 4 to 5 days after inoculation, seedlings at 20, 25 and 35°C also developed downy mildew symptoms. Seedlings at 15°C remained symptom-less for 8 days, but when they were moved to 20°C, they developed >75 per cent downy mildew within 5 days. Downy mildew did not develop at 40 and 45°C up to 13 days after inoculation (Singh and Gopinath, 1990).

Dew formation was the most probable variable that could be useful in predicting the downy mildew incidence of pearl millet. In addition, relative humidity and maximum temperature were also found to be positively correlated with the disease incidence (Muthusamy, 1979). Light was not found to affect zoospore infectivity (Singh and Gopinath, 1990). Distribution of rainfall was important in the expression of downy mildew (Nene and Singh, 1976), rather than the total rainfall (Suryanarayana, 1965).

Effect of different environmental factors such as temperature, light, relative humidity, and dew formation on germination of sporangia/conidia, and infectivity of different downy mildew pathogens were studied in several other crops such as sorghum (Safeeulla *et al.*, 1974; Schmitt and Freytag, 1974; Schuh *et al.*, 1987), maize (Chang and Wu, 1969; Exconde *et al.*, 1967; Bonde and Melching, 1979; Bustaman and Kimiga Fukuro, 1981; Bonde *et al.*, 1992), Chinese cabbage (Shao *et al.*, 1990), cucurbits (TSai and HSu, 1989), alfalfa (Patel, 1926; Melhus and Patel, 1929; Fried and Stuteville, 1977), soybean (Kirik *et al.*, 1985), peas (Singh *et al.*, 1988) and onion (Hilderbrand and Sutton, 1984a,b).

2.6 Mechanisms of disease resistance in plants

Resistance in plants to diseases is expressed through certain anatomical features, physiological functions, or chemicals .

2.6.1 Anatomical characters of leaf in crop plants with respect to disease resistance

The first line of defense against pathogens is the surface barriers which a pathogen must overcome before it can cause infection. The entry of the pathogen may either be through the epidermal cell walls, directly, or through the natural openings, or through injured areas caused by living or non-living agents. Certain structural features of the epidermis, or its interior, may greatly affect the ability of the pathogen to penetrate. The literature available on this aspect on some diseases is reviewed under the following headings.

A. Cuticle and epidermis

The cuticle forms the outer most covering of the epidermal cells and appears as a non-cellular, membranous layer. The cuticle acts as a physical barrier to penetration. The thick and tough outer wall of epidermal cells forms an important barrier for certain pathogens. Hawkins and Harvey (1919) found that potato tubers resistant to *Pythium debaryanum* have a tough epidermis with higher fibre content than the tubers of susceptible varieties.

Melander and Craigie (1927) attributed disease resistance to *Puccinia graminis*, in *Berberis spp.*, to the outer epidermal cell wall with a thicker cuticle. Similar results were reported in case of *Colletotrichum coffeanum* resistance in coffee (Nutman and Roberts, 1960), *Sphaerotheca macularis* resistance in

strawberries (Peries, 1962), *Botrytis cineria* resistance in beans, tomatoes and other plants (Louis, 1963), *Rhizoctonia solani* resistance in beans (Stockwell and Hanchey, 1983), *Cercospora personatum* resistance in groundnut (Jasbir Kaur and Dhillon, 1988), and *Cerotelium fici* resistance in mulberry (Tomy Philip *et al.*, 1991). Sharvelle (1936) pointed out that a strong epidermal membrane might limit the ability of a fungus to break out of diseased plant and reduce the available inoculum.

The cutin content in the epidermis was higher in *Allium galanthum* lines resistant to downy mildew (*Peronospora destructor*) than in the epidermis of susceptible line (Taleiva and Furst, 1985). Pavlenko (1993) also observed that plants of *Allium spp.*, resistant to downy mildew had a well developed cuticle, with pronounced layers. They also reported lower pectin content but higher phospholipids content in the cell walls of the epidermal tissue of resistant plants than in the tissues of susceptible plants.

However, the cases of absence of any relationship between the infectivity and cuticle thickness have also been reported. Johnstone (1931), for example, concluded that anatomical barriers are of secondary importance for scab resistance in apple. Also, resistance to powdery mildews was found independent of morphological or chemical differences in the cuticles of leaves of barley (Mackie, 1928), wheat (Smith and Blair, 1950), lettuce (Schnathorst, 1959), apple or rose (Roberts *et al.*, 1961) and peach (Weinhold and English, 1964).

Almost similar conclusions were drawn in cases of powdery mildew resistance in *Euonymus japonicus* (Roberts *et al.*, 1961), *Pseudocercosporella herpotrichoides* resistance in winter wheat (Murray and Bruehl, 1983), and *Pyricularia oryzae* resistance in rice (Veeraraghavan, 1983).

Berry (1959), in an investigation on onion downy mildew, found no barrier structures in the epidermis that might be associated with resistance, immunity could be broken down by maintaining moisture on the surface of plants under field conditions. Meherunnisa Begum (1984) reported that the thickness of the leaf epidermis does not have a role to play in resistance and susceptibility of pearl millet cultivars resistant and susceptible to downy mildew.

B. Leaf surface waxes

Leaf surface waxes were found to play an important role in the resistance mechanism against several diseases. For example, apple leaf wax was found to be toxic to mildew (Martin *et al.*, 1957) and a 40-70 per cent reduction in conidial germination of *Botrytis cinerea* was observed when grown in wax obtained from beet root leaves (Blakeman and Sztejnberg, 1973). Cruickshank *et al.* (1977) reported the inhibition of germination of *Peronospora hyoscyami* on *Nicotiana debneyi* leaves which are known to contain higher levels of duvatrienediols (waxes). Greater infection of *Albugo candida* on Brusselsprout leaves was observed in the absence of wax (Wilson and Jarvis, 1963). In gymnosperms too,

inhibition of fungal penetration was reported due to the deposition of wax on ginkgo leaves (Johanston and Sproston, 1965).

The wax content was high in the wheat varieties resistant to *Alternaria triticina* and low in the susceptible one (Kumar, 1974). Similar results were reported in cases of tikka disease resistance in groundnut (Gupta *et al.*, 1985) and *A. sesami* resistance in sesame (Gupta *et al.*, 1987).

C. Structure of natural openings

Most pathogens enter plants through natural openings. Pool and Mckay (1916) found that the germ tubes of *Cercospora beticola* could enter sugarbeet leaves only through open stomata. In case of stem rust of wheat caused by *Puccinia graminis tritici*, the varieties in which stomata open late in the morning are resistant because the germ tubes desiccate as the dew evaporates (Hart, 1929).

Tomy Philip *et al.* (1991) reported that the stomatal frequency was significantly higher in mulberry varieties susceptible to *Cerotelium fici* than in the resistant varieties.

Brahmachari and Kolte (1983) observed less number of stomata in groundnut varieties resistant to *Cercospora* leaf spot compared with susceptible varieties. Jasbir Kaur and Dhillon (1988) also reported that the size, frequency and index of stomata are significantly higher in susceptible varieties. Similarly, the rust

susceptible genotypes of wheat had larger stomata and more number of stomata per unit of leaf area than the resistant genotypes (Sokhi *et al.*, 1985).

On the other hand, Subramanyam *et al.* (1982) found no relationship between rust resistance and number of stomata on groundnut leaf, as the germ tubes can penetrate through the closed stomata. Meherunnisa Begum (1984) found that the number of stomata present on leaf surface does not have a direct relationship with the resistance or susceptibility in pearl millet cultivars resistant and susceptible to downy mildew.

2.6.2 Physiology and biochemistry of resistance in plants with respect to downy mildew

The physiology and biochemistry of plants altered by infection with downy mildew pathogen has been studied by some workers. The literature available on this aspect is reviewed under the following headings.

A. Changes in carbohydrates

In general, obligate pathogens are considered as high sugar diseases (Allen, 1959). Alagianagalingam *et al.* (1978b) and Muthusamy (1979) observed more total soluble sugars in the leaves of susceptible pearl millet cultivar than in corresponding tissues of the resistant cultivars. They opined that this may be related to the greater infection by *S. graminicola*. Similar results were reported in

sorghum (Basarkar *et al.*, 1988-1990) and onion (Taleiva and Furst, 1985). Conversely, Chahal *et al.* (1978) reported higher levels of carbohydrates in leaves of 40-day-old plants of resistant pearl millet variety, PHB 14, than in susceptible NHB3. On the other hand, Parashar *et al.* (1987) and Rajaram Reddy (1978) found that total sugars and reducing sugars did not differ in healthy leaves of resistant and susceptible pearl millet cultivars. The results of Luthra *et al.* (1988) in downy mildew of lucerne are also in agreement with this.

Gupta and Gupta (1984) observed decrease in reducing sugars and increase of non-reducing sugars in infected leaves of the resistant pearl millet cultivar, BJ 104, while the reverse trend was observed in the susceptible cultivar, HB 3. Muthusamy (1979) found that the inoculation caused increase of total soluble sugars in early stages and decrease in later stages in resistant varieties of pearl millet while the increase was seen throughout in the susceptible varieties. However, Mogle and Mayee (1981) reported no alterations and slight changes in a resistant and a moderately resistant line, respectively. They also observed increased reducing sugars and decreased starch content in a susceptible line.

In sorghum, both the resistant and susceptible cultivars were found to contain increased reducing sugar content and decreased total sugars and starch due to infection (Anwar *et al.*, 1989).

B. Changes in phenolic compounds

Host plant resistance to several pathogenic fungi has been ascribed to higher concentrations of phenolic substances and their oxidative products. Alagianagalingam *et al.* (1978a) observed that the leaves of pearl millet varieties resistant to *S. graminicola* contained more total phenols than those of susceptible variety HB 3. Chahal *et al.* (1978) also found higher levels of phenolic compounds in leaves of 40-day-old plants of resistant pearl millet variety PBH-14 than in susceptible NHB-3. Satija *et al.* (1983) reported that the phenolic content is linearly related to the degree of resistance at both the 30-and the 50-day growth stages. Similarly, Parashar *et al.* (1987) observed higher levels of phenolic compounds in resistant cultivars.

Muthusamy (1979) observed that the total and O-dihydroxy phenol contents accumulated more with infection. The inoculation increased accumulation in the initial stages and reduced at later stages of infection in susceptible varieties, while in resistant varieties, there was no reduction in the content of bound phenols due to infection. Similarly, Shekawat and Arya (1979) reported that hyperauxiny and increased levels of total and O-dihydroxy phenolics were associated with abnormal ear head of pearl millet. Kumhar *et al.* (1990) observed that the total phenol contents were high in shoots and roots of pearl millet infected by *S. graminicola.*

The correlation between higher levels of phenols and resistance to downy mildew pathogen has been reported in other crops such as sorghum (Shetty and Ahmad, 1980); lucerne (Luthra *et al.*, 1988); onion (Taleiva and Furst, 1985) and grapevine (Marutyan *et al.*, 1979).

C. Changes in Oxidative enzymes

Polyphenol oxidase (PPO) and Peroxidase (PO) activity was highest in roots and leaves of pearl millet varieties resistant to downy mildew (Alagianagalingam *et al.*, 1978a). They suggested that the defence mechanism produced by the oxidation of phenolics into highly reducing quinones may be operative in pearl millet. Satija *et al.* (1983) reported that PPO activity was linearly proportional to resistance. Similarly, Thukral *et al.* (1986) found that the activities of PPO and PO were positively correlated with resistance to *S. graminicola*.

Kumar *et al.* (1987) found that presence or absence of cathodal band (C2) and an anodal band (A4) in the zymogram of PO were associated with the degree of resistance to downy mildew. Chahal *et al.* (1988) suggested possible involvement of an isoperoxidase (C9) in imparting the resistance. The involvement of 2 other isozymes (C5 and C6) was also indicated in the resistance mechanism. They thought that differentiation in particular isozyme patterns may be a useful criterion for the characterization of resistance to downy mildew.

However, Muthusamy (1979) reported that there was no correlation between PO and PPO activity and downy mildew resistance. Rao (1983) also did not observe any differences in the PO isozyme patterns in dry, imbibed and germinated seeds of resistant and susceptible pearl millet cultivars.

The activity of oxidative enzymes, PO and PPO, was increased after inoculation in four genotypes of pearl millet, irrespective of their level of susceptibility to *S. graminicola* (Muthusamy, 1979). Shekawat and Arya (1979) observed increased activity of PO in diseased ear heads compared to normal ones, but the reverse trend was observed with Indole Acetic Acid Oxidase (IAAO) and PPO. Arora *et al.* (1986) also observed higher activity of PO in infected plants than in healthy plants. They reported that IAAO activity was also higher in infected plants at the seedling, boot and flowering stages but not at the dough stage. However, Thukral *et al.* (1986) reported that the activity of PO and PPO did not differ between healthy and infected tissues of the same genotypes. Rao (1983) also did not observe any differences on the isoperoxidase patterns of the healthy and infected seedlings of pearl millet.

The correlation between oxidative enzymes and resistance to downy mildew has been reported in sorghum (Gowda *et al.*, 1989), onion (Talieva and Furst, 1985), tobacco (Edreva *et al.*, 1983/84; Ye *et al.*, 1990a,b; Wyatt *et al.*, 1991; Gonzalez *et al.*, 1992) and grape vine (Marutyan *et al.*, 1979; Calderon *et al.*, 1992).

D. Changes in amino acids

Some of the amino acids, which have a central position in the nitrogen metabolism of plants such as glycine, asparagine, aspartic and glutamic acids, have been found to increase susceptibility to fungal diseases (Van Andel, 1966). Muthusamy (1979) found free amino acids viz., arginine, glycine, glutamic acid, and lucine in higher concentration in the highly susceptible variety of pearl millet. Conversely, Chahal *et al.* (1978) observed higher levels of amino acids in leaves of 40-day-old plants of downy mildew resistant variety, PHB-14, than in susceptible, NHB-3. However, Rao (1983) found no quantitative differences in levels of free amino acids in the root exudates of pearl millet cultivars resistant and susceptible to *S. graminicola*. Thakur and Murty (1984) also reported similar results.

Total free amino acids were considerably reduced by infection in early growth, the changes being more marked in the susceptible line (Mogle and Mayee, 1981). Kumhar *et al.* (1990) also reported that total free amino acids were low in shoots and roots of pearl millet infected by *S. graminicola*. Similar observations were recorded by Anwar *et al.* (1990) in sorghum and maize infected with *P. sorghi*.

Frost hardy grapevine hybrids, highly resistant to *Plasmopara viticola*, showed a marked decrease in the content of amino acids. In susceptible hybrids, there was an increase favouring development of the pathogen (Marutyan *et al.*, 1979). Leaves and shoots of healthy grapevine hybrids susceptible to *P. viticola*

contained relatively high amounts of aspartic and glutamic acids. In infected susceptible hybrids, the content of these acids decreased where as in resistant hybrids it increased. The amount of lysine and arginine was higher in healthy susceptible hybrids than in resistant ones and then doubled in leaves of susceptible hybrids, 13-15 days after infection (Antonyan and Marutyan, 1984a).

E. Changes in Proteins

In addition to the phenolic substances, produced in plant tissues in response to infection, induced synthesis of proteins seems to play a role in disease resistance. Seeds of the pearl millet cultivar BJ 104, resistant to *S. graminicola*, had less crude protein content than those of the susceptible cultivar HB 3 (Gupta and Gupta, 1984). Similarly, Taleiva and Furst (1985) observed that the tissues of vegetative organs of *Allium galanthum* lines resistant to *P. destructor* had lower contents of the total proteins and protein nitrogen. The increase in b-proteins coincided with the onset of immunization in tobacco plants injected with *Peronospora tabacina* and the levels were maintained during the period, after challenge, when the development of *P. tabacina* was restricted (Tuzen *et al.*, 1989).

In peas, electrophoretic analysis showed that anode globulins and cathode albumins from aleurone of grains and cathode globulins and albumins of the embryo were indicators of resistance to *P. pisi* (Volodin and Timofev, 1984). Total protein content was increased in the infected zone (Singh *et al.*, 1993).

Maccinkowsca *et al.* (1984) also observed higher protein content in soybean seeds infected by *P. manshurica* than in healthy seeds. The grape hybrids, resistant and susceptible to *Plasmopara viticola*, had similar protein contents when healthy. The protein synthesis in the shoots of resistant hybrids increased with infection but decreased in the shoots of susceptible hybrids. Susceptible and resistant hybrids showed different reaction as infection progressed in terms of content of individual proteins. For instance, at the 3rd day of infection, albumin content decreased in resistant but rose in susceptible hybrids while the reverse was true of lipoprotein content (Antonyan and Marutyan, 1984b).

F. Changes in ascorbic acid content

Jain and Arya (1978) demonstrated that healthy leaves of pearl millet contained almost twice as much ascorbic acid as those of infected ones (chlorotic ones), whereas healthy ears had half the amount of ascorbic acid found in proliferated green ears. It was postulated that under conditions of infection, the higher amounts of the ascorbic acid in ears stimulated nucleic acid, and protein metabolism, which increases cell division and enlargement resulting in the transformation of floral parts into leafy structures.

G. Changes in respiration

Mandahar and Garg (1974) calculated 149.45 per cent increase in the respiration in *S. graminicola* infected pearl millet leaves compared with healthy

ones. The normal respiratory path ways (EMP and TCA) were slightly enhanced, and an additional oxidative path way becomes operative in the diseased tissues. Infected leaves showed uncoupling of phosphorylation from oxidation and the operation of HMP pathway in them (Garg and Mandahar, 1975). Uncoupling has been assumed to be one of the causes of increased respiratory activity after infection.

H. Osmatic changes

Hegde and Karande (1978) pretreated seeds of pearl millet with varying osmatic concentrations of sodium chloride. It was observed that in the leaves, osmotic concentrations of the cell sap increased with the increasing concentrations of sodium chloride. With the increase in concentrations of the cell sap, in terms of osmatic pressure, the incidence of the disease decreased markedly. This was accomplished by a decrease in total carbohydrates, total nitrogen, proline, phosphorus and iron contents. With the decrease in disease index there was an increase in total chlorophyll and polyphenols of the leaves. It is inferred, therefore, that disease resistance could be induced by inducing high osmotic concentration of the cell sap.

MATERIALS AND METHODS

CHAPTER III

MATERIALS AND METHODS

The investigations on the mechanisms of resistance in pearl millet genotypes to downy mildew disease were carried out at the Asia Center of the International Crops Research Institute for the Semi-Arid Tropics (IAC), Patancheru, Andhra Pradesh, 502 324, India.

The materials and methods used in the present investigations are broadly described under the following heads:

1. General.
2. Germination of sporangia and zoospores on different parts of the second leaf of a resistant and a susceptible genotype.
3. Germination of sporangia and zoospores on the middle parts of the second leaf of resistant and susceptible genotypes.
4. Germination of sporangia and zoospores on 1st to 8th leaf of a resistant and a susceptible genotype.
5. Penetration of second leaf of resistant and susceptible genotypes by zoospore germ tubes.
6. Penetration of 1st to 8th leaf of a susceptible and a resistant genotype by zoospore germ tubes.

7. Effect of temperature on *in vivo* germination of sporangia and zoospores, and leaf penetration by germ tubes.
8. Colonization of 1st to 5th leaf by *Sclerospora graminicola* in resistant and susceptible genotypes.
9. Anatomical basis of resistance/susceptibility to the disease: the following three parameters were studied.
    (i) Thickness of cuticle.
    (ii) Epicuticular waxes.
    (iii) Number of stomata.
10. Biochemical basis of resistance/susceptibility to the disease: the analysis was made for the following compounds.
    (I) Total Phenols.
    (Ii) Total soluble sugars.
11. Statistical analysis.

3.1 General

Common materials and methodologies are described in this section. The finer details whereever needed are given in the respective sections.

3.1.1 Plant material

Two susceptible (HB 3 and 7042 S) and seven resistant (IP 18292, IP 18293, IP 18294, IP 18296, 7042 RR, P 310 and P 1449) pearl millet genotypes were used.

3.1.2 Maintenance of the host plants

Plants were grown in 12.5 cm diameter plastic pots, filled with a mixture of Alfisol and sand (2:1, v/v), in the greenhouse. The temperature in the greenhouse ranged from 25-30°C. To obtain seedlings in optimum health, seeds were surface sterilized, before sowing, with 5.25 per cent sodium hypochlorite (clorox) for 3 min followed by several rinses in distilled water. Plant protection measures were not used.

3.1.3 Preparation of inoculum

Sporangial inoculum was obtained from the systemically infected leaves (Plate 1) of 7042 S or HB 3 plants, inoculated by the ICRISAT isolate of the pathogen. Leaves were thoroughly washed with moist cotton to remove old downy growth of the pathogen. The leaves were cut into small pieces, which were kept in humidity chambers made by lining both the lids of plastic trays with moist filter paper. The humidity chambers were incubated in dark at 20°C for 6 hours (h) for sporulation. After this incubation period, incubator was programmed to a temperature of about 5°C in order to prevent sporangial germination. Sporangia were collected by washing the leaves with cold (5-6°C) distilled water. The

Plate 1: Systemic infection of downy mildew on a pearl millet leaf.

concentration of sporangia was estimated using a haemocytometer and adjusted to a desired concentration.

3.1.4 Microscopes

(i) Olympus BH-2 light microscope

(ii) Olympus Vanox Fluorescence microscope

3.1.5 Glassware

"Borosil", "Corning" and "Pyrex" brand glassware were used.

3.1.6 Chemicals

In all the experiments, AR grade chemicals of BDH, E. Merck and Sigma were used.

3.2 Germination of sporangia and zoospores on different parts of the second leaf of a resistant and a susceptible genotype.

3.2.1 Raising seedlings

Seeds of a susceptible (HB 3) and a resistant (IP 18293) genotype were planted in 12.5 cm diameter plastic pots on the same day to obtain seedlings with the fully-expanded second leaf at a time. A genotype was planted in two pots and there were about five seedlings per pot.

3.2.2 Inoculation

The second, fully-expanded, intact leaves were used. The leaves of the potted seedlings were maintained in a horizontal position to keep the inoculum stationary on the adaxial surface. Viable sporangia suspended in water ($1x10^4$ sporangia ml^{-1}) were sprayed on the adaxial surface of the leaves (Plate 2). The seedlings were covered with wet polythene bags and incubated at $20\pm1°C$ and $\geq$ 95 per cent Relative Humidity (RH) in dark for two and half hours.

3.2.3 Collection of leaf samples

The tip, middle, and basal parts of the leaf were collected, two and half hours after inoculation (HAI). Leaf samples from the resistant and susceptible genotypes were collected separately.

3.2.4 Processing of leaf samples

The leaf samples were processed following the method of Daimacio and Exconde (1969-70), with some modifications.

3.2.4.1 Chemicals used

(i) Glacial acetic acid

(ii) Ethanol (99.9 per cent)

(iii) Lactophenol

Lactic acid	20 ml
Phenol crystals	20 g

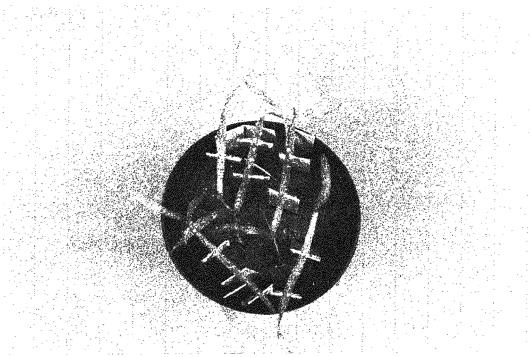

Plate 2: Spray inoculation of S. graminicola sporangia on intact leaves of pearl millet seedlings.

Glycerine	40 ml
Distilled water	20 ml

3.2.4.2 Stain used

Trypan blue (0.2 per cent) : Trypan blue (0.2 g) was dissolved in 100 ml of distilled water.

Fungiflour (solution B, Polysciences Inc. Warrington) and 0.2 per cent aniline blue (1:1, v/v) solution.

3.2.4.3 Fixation and clearing of leaf samples

Leaf samples (1 cm long) were fixed in a fixative containing glacial acetic acid and ethanol (1:2, v/v). The samples were kept in this solution for 6 to 16 h at 60°C. The fixative was decanted and the leaf samples were transferred to lactophenol for clearing at 60°C for 4 to 16 h. The samples can be stored in lactophenol indefinitely at room temperature (28±2°C).

3.2.4.4 Staining

After clearing, the lactophenol was decanted. The samples were washed with distilled water, and stained with trypan blue (0.2 per cent) for 30 minutes (min). Excess stain was removed by washing the leaf samples with distilled water and observed under light microscope (or) the samples were stained with fungiflour (solution B, Polysciences Inc. Warrington) and 0.2 per cent aniline blue (1:1, v/v) solution and observed under fluorescence microscope.

3.2.5 Microscopic examination

For each treatment, and for each genotype, ten leaf samples were observed for germination of sporangia and zoospores. Examination was done under light microscope (250 X magnification) or under fluorescence microscope (168 X magnification). A total of about 500 sporangia and 500 zoospores were observed for each treatment in 10 leaf samples @ 50 sporangia and 50 zoospores on each leaf sample. Per cent germination was calculated for both sporangia and zoospores.

3.3 Germination of sporangia and zoospores on the middle parts of the second leaf of resistant and susceptible genotypes

3.3.1 Raising seedlings

Seeds of nine genotypes, two susceptible (HB 3 and 7042 S) and seven resistant (IP 18292, IP 18293, IP 18294, IP 18296, 7042 RR, P 310, and P 1449) were planted in 12.5 cm diameter plastic pots on the same day to obtain seedlings with fully-expanded second leaves at a time. The pots were maintained in the greenhouse. A genotype was planted in two pots and there were about five seedlings per pot.

3.3.2 Inoculation

Inoculation was done as described in section 3.2.2.

3.3.3 Collection, processing, and microscopic examination of leaf samples

The leaf samples (2 cm long) were collected two and half HAI. The leaf samples were fixed, cleared, and stained as described in section 3.2.4. The samples were observed under the light microscope (250 X magnification) or under fluorescence microscope (168 X magnification). A total of about 500 sporangia and 500 zoospores were observed for each treatment @ 50 sporangia and 50 zoospores on each leaf sample. Per cent germination was calculated for both sporangia and zoospores.

3.4 Germination of sporangia and zoospores on 1st to 8th leaf of a resistant and a susceptible genotype

3.4.1 Raising seedlings

Staggered planting of a susceptible (HB 3) and a resistant (IP 18293) genotype was done in 12.5 cm diameter plastic pots, in the greenhouse, to obtain seedlings with fully-expanded 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 8th leaf stages at a time. Five seedlings per pot were maintained for 1st, 2nd, and 3rd leaf stages and there were about two pots for each leaf stage and for each genotype. Two to three seedlings per pot were maintained for 4th, 5th, 6th, 7th, and 8th leaf stages and there

were about 3 to 5 pots for each leaf stage and for each genotype. At least 10 seedlings with desired leaf stage were maintained.

3.4.2 Inoculation

Inoculation was done as described in section 3.2.2.

3.4.3 Collection, processing, and microscopic examination of leaf samples

Two-and-half HAI, leaves from the eight growth stages (1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 8th leaf stages), and from both the susceptible and resistant genotypes, were detached separately, and cut into small pieces (2 cm long). The samples were fixed, cleared, and stained as described in section 3.2.4. Ten leaf samples from each growth stage from both the resistant and the susceptible genotypes, were observed under light microscope (250 X magnification) or under fluorescence microscope (168 X magnification). A total of about 500 sporangia and 500 zoospores were observed for each treatment in 10 leaf samples @ 50 sporangia and 50 zoospores on each leaf sample. Per cent germination was calculated for both sporangia and zoospores.

3.5 Penetration of second leaf of resistant and susceptible genotypes by zoospore germ tubes

3.5.1 Raising seedlings

Seeds of two susceptible (HB 3 and 7042 S) and four resistant (IP 18292, IP 18293, IP 18294, and IP 18296) genotypes were planted in 12.5 cm diameter plastic pots on the same day to obtain seedlings with fully-expanded second leaves at one time. A genotype was planted in six pots. There were about five seedlings per pot. The pots were maintained in the greenhouse.

3.5.2 Inoculation

Inoculation was done as described in section 3.2.2.

3.5.3 Collection of leaf samples

The middle parts of leaves (2 cm long) were collected 2, 4, and 6 HAI from both the resistant and susceptible genotypes separately.

3.5.4 Processing of leaf samples

3.5.4.1 Fixation and clearing

The leaf samples were fixed and cleared as described in section 3.2.4.3.

3.5.4.2 Staining

After clearing, the lactophenol was decanted. The samples were washed with distilled water, and stained with fungiflour (solution B, Polysciences

Inc. Warrington) and 0.2 per cent aniline blue (1:1, v/v) solution to observe under fluorescence microscope. Samples were also stained with trypan blue (0.2 per cent) to observe stomatal penetration under light microscope.

3.5.5 Microscopic examination

Ten leaf samples from both the susceptible and resistant genotypes were observed at each sampling time under fluorescence microscope (168 X magnification). Samples were also observed under light microscope (250 X) for stomatal penetration. A total of about 500 germinated zoospores were observed for each treatment in 10 leaf samples @ 50 germinated zoospores on each leaf sample and per cent penetration was calculated.

3.6 Penetration of 1st to 8th leaf of a susceptible and a resistant genotype by zoospore germ tubes

3.6.1 Raising seedlings

Staggered planting of a susceptible (HB 3) and a resistant (IP 18293) genotype was done in 12.5 cm diameter plastic pots, in the greenhouse, to obtain seedlings with fully-expanded 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 8th leaf stages at a time. Five seedlings per pot were maintained for 1st, 2nd, and 3rd leaf stages and there were about two pots for each leaf stage and for each genotype. Two to three seedlings per pot were maintained for 4th, 5th, 6th, 7th, and 8th leaf stages and there

were about 3 to 5 pots for each leaf stage and for each genotype. At least 10 seedlings with desired leaf stage were maintained.

3.6.2 Inoculation

Inoculation was done as described in section 3.2.2

3.6.3 Collection of leaf samples

The middle parts of leaves (2 cm long), were collected 6 HAI.

3.6.4 Processing of leaf samples and microscopic examination

The leaf samples were processed as described in section 3.5.4. Ten samples for each leaf stage, for both resistant and susceptible genotypes, were observed under fluorescence microscope (168 X magnification). A total of about 500 germinated zoospores were observed for each treatment in 10 leaf samples @ 50 germinated zoospores on each leaf sample for penetration and per cent penetration was calculated.

3.7 Effect of temperature on *in vivo* germination of sporangia and zoospores, and leaf penetration by germ tubes

3.7.1 Raising seedlings

Seeds of a susceptible (HB 3) and a resistant (IP 18293) genotype were planted on the same day to obtain seedlings with the fully-expanded second leaf

at a time. Each genotype was planted in twenty, 12.5 cm diameter plastic pots. Five seedlings were maintained in each pot.

3.7.2 Inoculation

The leaves of the potted seedlings were maintained in a horizontal position to keep the inoculum stationary on the adaxial surface. The adaxial surfaces of leaves were spray-inoculated with viable sporangial suspension ($1x10^4$ sporangia ml^{-1}) (Plate 2). Immediately after inoculation, plants were covered with wet polythene bags and incubated at five temperatures namely 15, 20, 25, 30 and 35°C, at $\geq$ 95 per cent RH in dark.

3.7.3 Collection of leaf samples

The leaf samples were collected two-and-half HAI to observe germination of sporangia and zoospores and at 6 HAI to observe the penetration by zoospore germ tubes.

3.7.4 Processing of leaf samples

The leaf samples were fixed, cleared and stained as described in section 3.5.4.

3.7.5 Microscopic examination

Ten leaf samples, for each sampling time, genotype, and incubation temperature, were examined under fluorescence microscope (168 X). A total of about 500 sporangia and 500 zoospores were observed for each treatment in 10

leaf samples @ 50 sporangia and 50 zoospores on each leaf sample and per cent germination was calculated. A total of about 500 germinated zoospores were observed for each treatment in 10 leaf samples @ 50 germinated zoospores on each leaf sample for penetration, and per cent penetration was calculated.

3.8 Colonization of 1st to 8th leaf by *S. graminicola* in resistant and susceptible genotypes

3.8.1 Raising seedlings

Staggered planting of two susceptible (HB 3 and 7042 S) and four resistant (IP 18292, IP 18293, IP 18294, and IP 18296) genotypes was done in 12.5 cm diameter plastic pots, in the greenhouse, to obtain seedlings with fully-expanded 1st, 2nd, 3rd, 4th, and 5th leaf stages at a time. Five seedlings per pot were maintained for 1st, 2nd, and 3rd leaf stages and there were about two pots for each leaf stage and for each genotype. Two to three seedlings per pot were maintained for 4th, and 5th leaf stages and there were about 3 to 5 pots for each leaf stage and for each genotype. Seedlings with desired leaf stage (50 seedlings for each leaf stage and for each genotype) were maintained.

3.8.2 Inoculation

The leaves of the potted seedlings were maintained in a horizontal position to keep the inoculum droplet stationary on the adaxial surface. In the middle part of the leaves, areas (1 cm long for 1st leaf and 2 cm long for the other

leaves) were marked with a marker pen. The inoculation was done on the marked areas on the adaxial leaf surface with a viable suspension of sporangia (1×10^4 sporangia ml^{-1}) using a microsyringe (Plate 3). Immediately after inoculation, plants were covered with wet polythene bags and incubated at 20°C and $\geq$ 95 per cent RH in dark for 24 h. Later, the plants were shifted to greenhouse at temperature ranging from 25 to 30°C.

3.8.3 Collection of leaf samples

The leaf samples were collected 24, 48, 72, 96 and 120 HAI.

3.8.4 Processing of leaf samples

3.8.4.1 Stain used

Cotton blue (0.1 per cent): It was prepared by dissolving 0.1 g cotton blue in 100 ml of distilled water.

3.8.4.2 Fixation and clearing

The samples were fixed and cleared as described in section 3.2.4.3.

3.8.4.3 Staining

After clearing, the lactophenol was decanted and after 4 to 5 washes in distilled water, the samples were stained with cotton blue (0.1 per cent) for 8 to 72 h depending upon the age of the leaves. Later, the samples were washed with distilled water and observed under light microscope (250 X magnification).

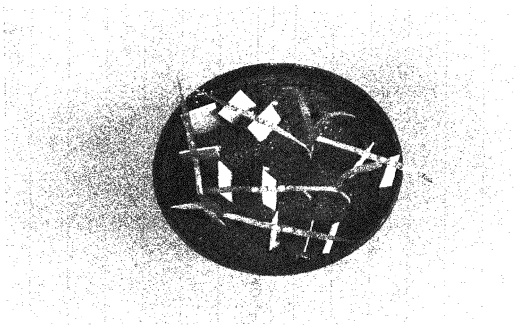

Plate 3: Drop inoculation of *S. graminicola* sporangia on intact leaves of pearl millet seedlings.

3.8.5 Microscopic examination

Ten samples for each leaf stage, for each sampling time, and for both resistant and susceptible genotypes separately, were examined for the presence of mycelium under light microscope (250 X magnification).

3.9 Anatomical basis of resistance/susceptibility to the disease: the following parameters were studied

3.9.1 Thickness of cuticle

3.9.1.1 Raising seedlings

Staggered planting of two susceptible (HB 3 and 7042 S) and four resistant (IP 18292, IP 18293, IP 18294, and IP 18296) genotypes was done in 12.5 cm diameter plastic pots, in the greenhouse to obtain seedlings with fully-expanded 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 8th leaves at a time. Five seedlings were maintained for each leaf stage and for each genotype.

3.9.1.2 Microtome used

Leitz 1516 rotary microtome

3.9.1.3 Chemicals and stain used

(i) Glutaraldehyde (50 per cent)

(ii) Phosphate buffer (pH 6.8)

(iii) Methyl cellusolve (2-Methoxyethanol) (99.5 per cent)

(iv) Ethanol (99.9 per cent)

(v) Propanol (99.5 per cent)

(vi) n-butanol (99.5 per cent)

(vii) LKB 2218-500 HISTORESIN embedding kit (LKB-Produkter AB, Box 305, S-16126 Bromma Sweden):

Basic resin (Glycol methacrylate monomer)

Activator (Benzoyl peroxide)

Hardener (Derivative of barbituric acid)

(viii) Sodium carbonate (0.1 per cent)

(ix) Toluidine blue (0.5 per cent)

3.9.1.3.1 Preparation of phosphate buffer (pH 6.8)

The phosphate buffer containing solution A and solution B was prepared as follows:

Solution A : 0.2 M monobasic sodium phosphate (24 g NaH_2PO_4 in 1000 ml distilled water).

Solution B : 0.2 M dibasic sodium phosphate (28.4 g Na_2HPO_4 in 1000 ml distilled water).

Solution A (50 ml) and solution B (49 ml) were added in a 200 ml volumetric flask, diluted to 200 ml with distilled water, and the pH was adjusted to 6.8.

3.9.1.3.2 Preparation of glutaraldehyde

Three per cent glutaraldehyde fixative was prepared by adding 6 ml glutaraldehyde (50 per cent) to 100 ml volumetric flask and the volume was made up with phosphate buffer.

3.9.1.3.3 Infiltration solution

Basic resin	50 ml
Activator	0.5 g

3.9.1.3.4 Intermediate solution

Infiltration solution	1 part
Ethanol (99.9 per cent)	1 part

3.9.1.3.5 Embedding solution

Infiltration solution	15 ml
Hardener	1 ml

3.9.1.3.6 Toluidine blue (0.5 per cent)

Toluidine blue	0.5 g
Sodium carbonate (0.1 per cent)	100 ml

3.9.1.4 Preparation of leaf material

3.9.1.4.1 Fixation and dehydration of leaf samples

The samples from middle parts of fully-expanded 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 8th leaves of the six genotypes were cut into small pieces (0.5 cm long) with clean single edge industrial blades and processed following the procedure of Jensen (1962) with some modifications. The samples were fixed in small glass vials containing 3 per cent glutaraldehyde for 48 h at 4°C. The fixative was changed twice during this time period. After fixation, the fixing solution was decanted and replaced with the first dehydrating solution, methyl cellusolve. Samples were processed separately in succession in 99.5 per cent methyl cellusolve, 99.9 per cent ethanol, 99.5 per cent n-propanol, and 99.5 per cent n-butanol for 24 h each at 0 ± 2°C. The solvents were changed twice during the 24 h period. The samples were placed in a desiccator under vacuum (to help the solvents to penetrate into the samples) in open vials for 30 min at the beginning of the first change of each solvent. Samples can be stored in butanol indefinitely.

3.9.1.4.2 Infiltration and embedding of leaf samples

After dehydration, butanol was decanted and replaced with the intermediate solution. Samples were soaked in intermediate solution for 48 h at 4°C and in infiltration solution for 72 h at 4°C. The solutions were changed twice during the above period. The samples were placed in a desiccator under vacuum (to help the solutions to penetrate into the samples) in open vials for 30 min at the beginning of the first change of each solution. After infiltration, the samples were embedded in the embedding solution.

3.9.1.5 Sectioning

Sections (2 µm) of the resin-embedded material were cut with a dry glass knife. The sections were collected with forceps and placed on pre-cleaned glass slides. Then the sections were air-dried.

3.9.1.6 Staining

The air-dried sections were stained with toluidine blue (0.5 per cent) and heated slightly on hot plate for few seconds. The sections were air-dried after washing with distilled water.

3.9.1.7 Measurement of cuticle thickness

The slides thus prepared were mounted on the stage of Olympus model BH-2 compound microscope fitted with photo eye-piece (X 5) and objective lens (X 10) and intermediate magnification of 1.25 X. The image of the sections were photographed using 125 ASA Kodak black and white 35 mm negative. Positive prints were made at an enlargement of X 5 giving total magnification of 312.5. The cuticle thickness was measured using X 7 Bausch and Lomb micrometer. The readings so obtained were accordingly reduced to the objective size by dividing with the magnification factor and the thickness of cuticle was expressed in µm. All the measurements were made at the middle of the cell wall, thus avoiding the thickenings at the corners of the cells. Fifty readings were taken from five different leaves for each leaf stage and for each genotype.

3.9.2 Epicuticular waxes

3.9.2.1 Raising seedlings

Staggered planting of two susceptible (HB 3 and 7042 S) and four resistant (IP 18292, IP 18293, IP 18294, and IP 18296) genotypes was done in 12.5 cm diameter plastic pots, in the greenhouse to obtain seedlings with fully-expanded 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 8th leaf. Each genotype was planted in 10 pots @ 20 seedlings per pot to obtain seedlings with 1st leaf; 15 pots @ 10 seedlings per pot to obtain seedlings with 2nd leaf; 10 pots @ 10 seedlings per pot to obtain seedlings with 3rd leaf; 15 pots @ 4 seedlings per pot to obtain seedlings with 4th and 5th leaf and 10 pots @ 4 seedlings per pot to obtain seedlings with 6th, 7th, and 8th leaf. Seedlings with desired leaf stage were maintained.

3.9.2.2 Chemicals used

(i) Chloroform

(ii) Chromic acid reagent

The reagent was prepared by mixing 40 ml deionized water with 20 g powdered potassium dichromate. The resulting slurry was mixed vigorously with 1 L concentrated sulphuric acid and heated (below boiling) until a clear solution was obtained.

3.9.2.3 Estimation of epicuticular waxes by colorimetric method

Fully-expanded 1st, 2nd, 3rd, 4th, 5th, 6th, 7th, and 8th leaves of all the six genotypes, were excised from the main seedling. The epicuticular waxes were estimated by the colorimetric method of Adelina Ebercon *et al.* (1977) with slight modifications. A 1.5 g fresh leaf sample was taken separately for each treatment. Later, the samples were immediately immersed in 20-25 ml analar distilled chloroform for 15 seconds for extraction of waxes. The extract was filtered and evaporated on a boiling water bath until the chloroform was fully evaporated (testing by the lack of smell). After adding 5 ml of chromic acid reagent, samples were boiled for 30 min. After cooling the samples, 12 ml of deionized water was added and the samples were stirred on a vortex mixer.The samples were kept for several minutes for color development.The optical density of the samples was read at 590 nm, using Spectronic 21. The amount of wax was expressed as mg g^{-1} fresh weight of the sample.

3.9.3 Number of stomata

3.9.3.1 Raising seedlings

Seeds of two susceptible (HB 3 and 7042 S) and four resistant (IP 18292, IP 18293, IP 18294, and IP 18296) genotypes were planted on the same day to obtain the seedlings with fully-expanded second leaves. Each genotype was planted in two ,12.5 cm diameter plastic pots, in the greenhouse. There were about five seedlings per pot.

3.9.3.2 Preparation of material

The leaf samples were processed following the procedure of Dalmacio and Exconde (1969-70) with some modifications. Fully-expanded second leaves of all the six genotypes were fixed in glacial acetic acid and ethanol (1:2, v/v) solution for 6-16 h at 60°C. The fixative was decanted and the leaf samples were transferred to lactophenol for clearing at 60°C for 4 to 16 h. The samples can be stored in lactophenol indefinitely at room temperature (28±2°C).

3.9.3.3 Microscopic examination

After clearing, the lactophenol was decanted and after 4 to 5 rinses in distilled water, the leaf samples were observed under light microscope (312.5 X) for counting stomata, both on the upper and lower surface of the leaf. The number of stomata were counted in twenty microscopic fields per leaf sample, each side and in ten leaf samples for each genotype. The stomata were quantified per mm^2 leaf area.

3.10 Biochemical basis of resistance/susceptibility in resistant and susceptible genotypes

3.10.1 Raising seedlings

(i) Staggered planting of two susceptible (HB 3 and 7042 S) and four resistant (IP 18292, IP 18293, IP 18294, and IP 18296) genotypes was done in 12.5 cm diameter plastic pots, to obtain seedlings with fully-expanded 1^{st}, 2^{nd}, and

3rd leaf stages at a time, to study the total phenols and total soluble sugars (TSS) in disease free seedlings. A genotype was planted in 70 pots @ 25 seedlings per pot to obtain seedlings with 1st leaf stage; 70 pots @ 10 seedlings per pot to obtain seedlings with 2nd leaf stage; 25 pots @ 10 seedlings per pot to obtain seedlings with 3rd leaf stage. Seedlings with desired leaf stage were maintained.

(ii) Seeds of all the six genotypes were planted on the same day, in 17.5 cm diameter plastic pots, to obtain seedlings with fully-expanded first leaf stage. A genotype was planted in 70 pots @ 20 seedlings per pot. This was done to study the changes in total phenols and TSS in susceptible and resistant genotypes after inoculation with the pathogen.

3.10.2 Inoculation

The seedlings at the first leaf stage were spray-inoculated with a viable sporangial suspension ($1X10^4$ sporangia ml^{-1}).Immediately after inoculation, plants were covered with wet polythene bags and incubated at 20°C and $\geq$ 95 per cent RH in dark for 24 h. Later, the plants were shifted to greenhouse at 25-30°C for the expression of the disease symptoms.

3.10.3 Collection of leaf samples

(i) The samples of disease free seedlings, from all the six genotypes, were collected for the three leaf stages; 1st leaf fully-expanded (5 days after sowing), 2nd leaf fully-expanded (7 days after sowing), and 3rd leaf fully-expanded (10 days after sowing). The roots were detached and the composite samples were

made without separating them into shoots and leaves to study total phenols and TSS in disease free seedlings.

(ii) Composite leaf samples of third leaves from all the six genotypes were collected 9 days after inoculation (only the 3rd infected leaves of inoculated seedlings and corresponding leaves of non-inoculated seedlings) to study the changes in total phenols and TSS after inoculation with the pathogen.

3.10.4 Preparation of samples for analysis

The collected plant samples were dried in an hot air oven at 37°C and powdered with "Willey" mill.

3.10.5 Phenols

3.10.5.1 Reagents used

(A) Reagents used for the estimation of total phenols

(i) 1N Folin-Ciocalteau's reagent: 2 N Folin-Ciocalteau's reagent was diluted to 1N with water.

(ii) Twenty per cent sodium carbonate solution: Sodium carbonate (20 g) was dissolved in water and the volume was made up to 100 ml with water.

(B) Reagents used for thin layer chromatography

(i) Mixture of n-butanol, acetic acid, water (4:1:5, v/v ratio)

(ii) Silica gel with 13 per cent calcium sulphate - Silica gel G-60

(iii) Silver nitrate in acetone

One ml of saturated aqueous silver nitrate was slowly added to 20 ml acetone with stirring and the product was treated drop wise with water until the precipitate silver nitrate has just dissolved.

3.10.5.2 Estimation of total phenols

Defatted sample (0.5 g) was dissolved in 20 ml of 95 per cent ethanol. After shaking for 1 h, filtered through Whatman no.41 filter paper. Twenty ml of petroleum ether AR (60-80°C), was added to the filtrate and shaken well. After sometime, the upper layer was removed. Washing with petroleum ether was repeated 6 to 7 times, till the chlorophyll was removed. The remaining solution was evaporated and the volume was made up to 2 ml. After this, the aqueous layer was filtered through udy filter.

To 0.1 ml of the above extract, 0.9 ml of distilled water, 1 ml of Folin ciocalteau's reagent and 2 ml of sodium carbonate solution were added and the resultant was incubated for 15 min in a water bath at 60°C. After cooling it, the extract was filtered through Whatman No.41 filter paper. The volume was made up to 10 ml with distilled water and the absorbance was read at 560 nm using Spectronic 21 against a reagent blank (Kaluza *et al.*,1980).Tannic acid was used as standard. The values were expressed as per cent total phenols of the sample.

3.10.5.3 Thin layer chromatography (TLC)

TLC was conducted for the separation of phenols from disease free leaves (fully-expanded 1st leaf) of two susceptible (HB 3 and 7042 S)and two

resistant (IP 18292 and IP 18293) genotypes following the procedure of Stefanis and Ponte (1968) with some modifications. TLC plates were coated with 0.5 cm silica gel G-60 (40 g of Silica gel G-60 was mixed thoroughly in 100 ml distilled water for 5 to 7 min and the slurry was spread on the plates). The sample extracts (equivalent to 20 µg phenols) were spotted on TLC plates. During spotting, hot air blower was used for drying spots. The spotted plates were kept in the saturated TLC chamber for 5 h. The solvent system used was a mixture of n-butanol, acetic acid and water (4:1:5, v/v ratio). The plates were removed when the solvent front is 2.5 cm before end of the plate and the plates were dried with the help of hot air blower. The dried plates were sprayed with saturated silver nitrate in acetone and the plates were dried in an oven at 110°C for the development of bands.

3.10.6 Total soluble sugars

3.10.6.1 Reagents

(i) Eighty per cent ethyl alcohol: 800 ml of ethanol was mixed with water and made up to 1 L with water.

(ii) Five per cent phenol: 5 g phenol was dissolved in water and the volume was made up to 100 ml.

(iii) Ninety six per cent sulphuric acid (v/v) (specific gravity 1.84).

(iv) Glucose standard

3.10.6.2 Estimation of total soluble sugars

The defatted sample (250 mg) was extracted four times with 80 per cent hot aqueous ethanol and finally evaporated to minimum quantity. The volume was

made up to 100 ml with water. A 0.5 ml aliquot was pipetted out and the color developed with phenol-sulphuric acid reagent was read at 490 nm against a water blank (Dubois *et al.*, 1956). Glucose was used as standard. The values were expressed as per cent TSS of the sample.

3.11 Statistical analysis

The results were statistically analyzed for correlation coefficients, regressions and analysis of variance (Snedecor and Cochran, 1968). The statistical analysis was carried out using GENSTAT program. The graphics were prepared using Free-lance package.

RESULTS

CHAPTER IV

RESULTS

4.1 Histopathological studies

Germination of sporangia and zoospores, germ tube penetration, and colonization of leaves were studied by light and fluorescence microscopy. Examination of the leaf pieces, two and half hours after inoculation (HAI), showed that most of the sporangia were germinated. Sporangia mostly germinate indirectly by producing zoospores. Zoospores emerge through a pore produced by the opening of the operculum in the apical region of the sporangium. Occasionally, direct germination of sporangia by forming a germ tube has also been observed (Plate 4).

Zoospores encyst and adhere to the surface after initial period of motility. The encysted zoospores germinate by forming a germ tube (Plate 5). The tips of the germ tubes enlarge and form appressoria which were globose (Plate 6). Sometimes, germ tubes may not form appressoria and penetrate the leaf (Plate 7).

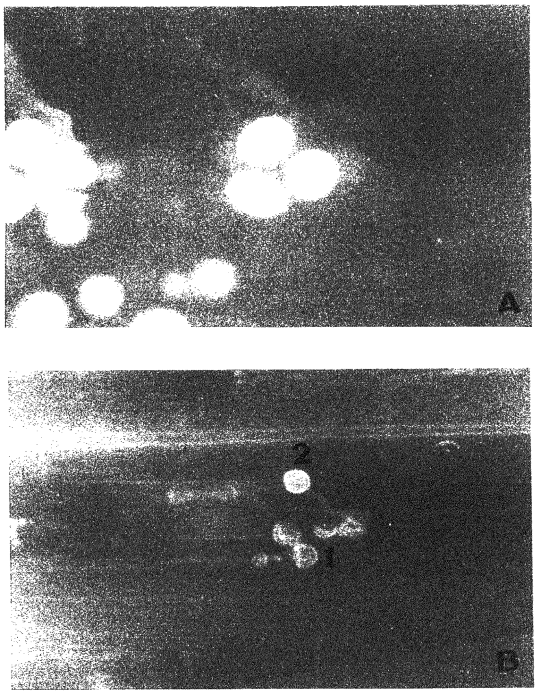

Plate 4: Fluorescence micrograph of S. graminicola sporangial germination on leaf surface of pearl millet.

A. Ungerminated sporangia (158.4 X)
B. 1. Empty sacs of sporangia after zoospore release (79.2X)
2. Direct germination of sporangia (79.2X).

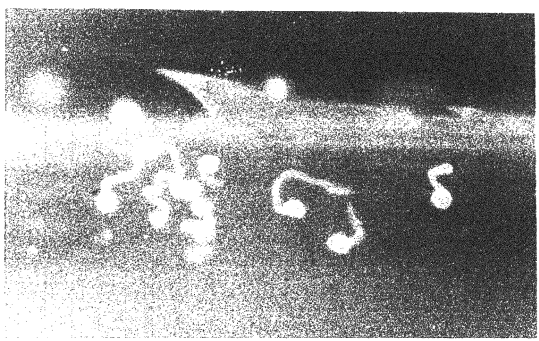

Plate 5: Fluorescence micrograph showing germinated zoospores of S. graminicola on the leaf surface of pearl millet (79.2 %).

Plate 6: Fluorescence micrograph showing appressoria at the tip of the zoospore germ tubes of S. graminicola on the lea. surface of pearl millet (79.2 X).

Plate 7: Fluorescence micrograph showing penetration of zoospore germ tubes of S. graminicola through the epidermal cells of pearl millet leaf without formation of appressoria (79.2 X).

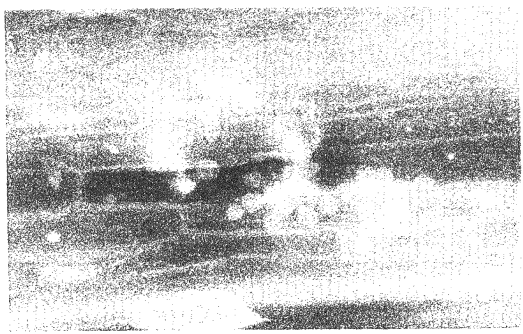

Plate 8: Fluorescence micrograph showing penetration of zoospore germ tubes of S. graminicola through the epidermal cells of pearl millet leaf after formation of appressoria (79.2 X).

Plate 7: Fluorescence micrograph showing penetration of zoospore germ tubes of S. graminicola through the epidermal cells of pearl millet leaf without formation of appressoria (79.2 X).

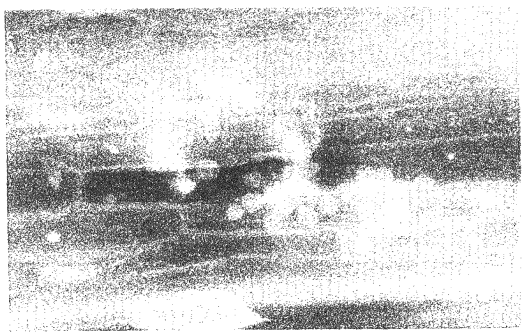

Plate 8: Fluorescence micrograph showing penetration of zoospore germ tubes of S. graminicola through the epidermal cells of pearl millet leaf after formation of appressoria (79.2 X).

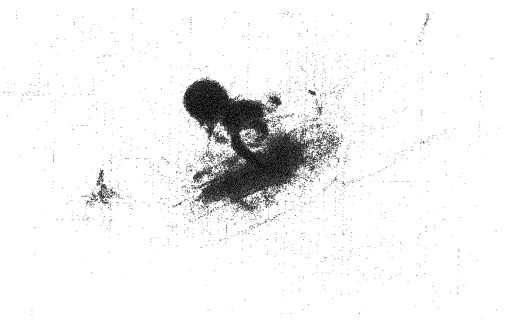

Plate 9: Light micrograph showing zoospore germ tube entering pearl millet leaf through stomata (250 X).

Table 1: Germination of sporangia and zoospores on different parts of the second leaf of a resistant and a susceptible genotype.

Part of the leaf	Sporangial germination (%)*		Zoospore germination (%)*	
	HB 3(S)	IP 18293(R)	HB 3(S)	IP 18293(R)
Tip	98.0 (82.03)	76.0 (60.68)	87.0 (68.87)	52.8 (46.61)
Middle	98.5 (83.12)	76.5 (61.01)	87.3 (69.14)	53.3 (46.89)
Base	98.7 (83.54)	77.0 (61.35)	88.0 (69.76)	54.0 (47.3)
Mean	98.4 (82.9)	76.5 (61.01)	87.43 (69.26)	53.37 (47.30)
Genotype				
SEM±	0.212 (0.234)		0.243 (0.170)	
CD at 5%	0.59 (0.65)		0.67 (0.47)	
Part of the leaf				
SEM±	0.260 (0.286)		0.298 (0.208)	
CD at 5%	0.72 (0.79)		0.83 (0.58)	
Genotype x part of the leaf				
SEM±	0.368 (0.405)		0.421 (0.294)	
CD at 5%	1.02 (1.12)		1.17 (0.81)	
CV (%)	1.3 (1.8)		1.9 (1.6)	

Figures in parenthesis are arcsin transformed values
* Mean of 10 leaf samples
S = Susceptible genotype; R = Resistant genotype.

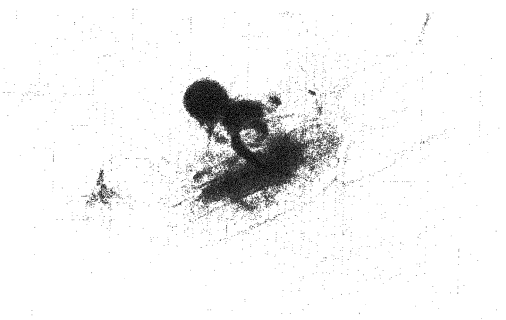

Plate 9: Light micrograph showing zoospore germ tube entering pearl millet leaf through stomata (250 X).

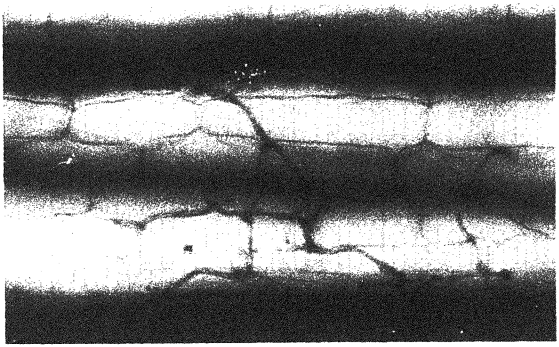

Plate 10: Light micrograph of S. graminicola mycelium in the epidermal layer of pearl millet leaf (125 X).

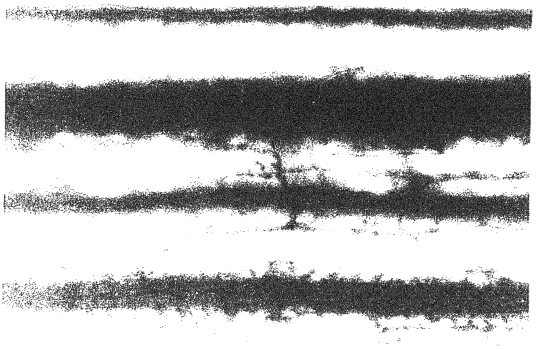

Plate 11: Light micrograph of S. graminicola haustoria in the epidermal layer of pearl millet leaf (125 X).

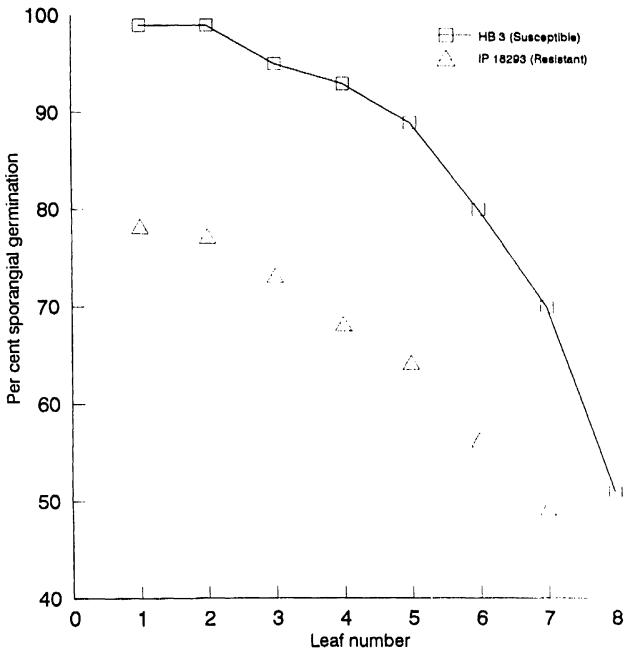

Fig. 1 : Germination of sporangia of *Sclerospora graminicola* on 1st to 8th leaf of a susceptible and a resistant genotype.

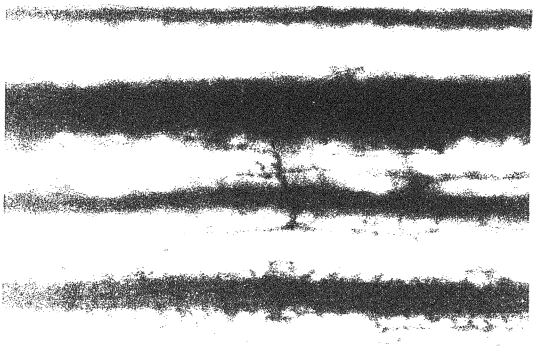

Plate 11: Light micrograph of S. graminicola haustoria in the epidermal layer of pearl millet leaf (125 X).

Table 1: Germination of sporangia and zoospores on different parts of the second leaf of a resistant and a susceptible genotype.

Part of the leaf	Sporangial germination (%)*		Zoospore germination (%)*	
	HB 3(S)	IP 18293(R)	HB 3(S)	IP 18293(R)
Tip	98.0 (82.03)	76.0 (60.68)	87.0 (68.87)	52.8 (46.61)
Middle	98.5 (83.12)	76.5 (61.01)	87.3 (69.14)	53.3 (46.89)
Base	98.7 (83.54)	77.0 (61.35)	88.0 (69.76)	54.0 (47.3)
Mean	98.4 (82.9)	76.5 (61.01)	87.43 (69.26)	53.37 (47.30)
Genotype				
SEM±	0.212 (0.234)		0.243 (0.170)	
CD at 5%	0.59 (0.65)		0.67 (0.47)	
Part of the leaf				
SEM±	0.260 (0.286)		0.298 (0.208)	
CD at 5%	0.72 (0.79)		0.83 (0.58)	
Genotype x part of the leaf				
SEM±	0.368 (0.405)		0.421 (0.294)	
CD at 5%	1.02 (1.12)		1.17 (0.81)	
CV (%)	1.3 (1.8)		1.9 (1.6)	

Figures in parenthesis are arcsin transformed values
* Mean of 10 leaf samples
S = Susceptible genotype; R = Resistant genotype.

Table 2: Germination of sporangia and zoospores on the middle parts of the second leaf of seven resistant and two susceptible genotypes.

Genotype	Sporangial germination (%)*	Zoospore germination (%)*
HB 3(S)	98.5 (83.12)	87.3 (69.14)
7042 S(S)	97.5 (81.11)	83.4 (65.97)
IP 18292(R)	61.3 (51.53)	53.0 (46.72)
P 1449(R)	55.8 (48.33)	55.7 (48.27)
IP 18293(R)	76.5 (61.01)	53.3 (46.89)
IP 18294(R)	86.1 (68.13)	59.2 (50.30)
IP 18296(R)	89.6 (71.24)	65.4 (53.97)
7042 RR(R)	93.2 (75.00)	57.8 (49.49)
P 310(R)	93.4 (75.17)	48.3 (44.02)
SEM±	0.453 (0.465)	0.540 (0.329)
CD at 5%	1.26 (1.29)	1.49 (0.92)
CV%	1.70 (2.2)	2.7 (2.0)

Figures in parenthesis are arcsin transformed values
* Mean of 10 leaf samples
S = Susceptible genotype
R = Resistant genotype

(IP 18293, IP 18294, IP 18296, 7042 RR and P 310). In general, zoospore germination was less than sporangial germination, irrespective of the susceptibility level of the genotypes.

4.4 Germination of sporangia and zoospores on 1st to 8th leaf of a resistant and a susceptible genotype

This experiment was conducted to determine the effect of leaves produced at different stages of plant growth, on germination of sporangia and zoospores. The results clearly show that the sporangial germination was nearly 100 per cent on first and second leaves, but then sharply decreased on the subsequent leaves, and on the eighth leaf, the germination was about 50.5 per cent on the susceptible genotype and 45 per cent on the resistant one (Table 3 and Fig. 1). This phenomenon is represented by fitting a quadratic curve, and the regression equations of the quadratic curve are given below.

$SG\ 1 = 93.45 + 5.27\ X - 1.289\ X^2\ (R^2 = 0.98)$

$SG\ 2 = 81.26 - 2.11\ X - 0.324\ X^2\ (R^2 = 0.99)$

SG 1 = Per cent sporangial germination on HB 3

SG 2 = Per cent sporangial germination on IP 18293

X = Leaf age

R^2 = Regression coefficient

Table 3: Germination of sporangia and zoospores of *S. graminicola* on 1st to 8th leaf of a susceptible and a resistant genotype.

Leaf No.	Days	Per cent sporangial germination* HB 3(S)	IP 18293(R)	Mean	Per cent zoospore germination* HB 3(S)	IP 18293(R)	Mean
1	5	99.0 (85.27)	78.0 (62.05)	88.5 (73.66)	88.2 (69.95)	55.4 (48.1)	71.8 (59.02)
2	7	98.5 (83.12)	76.5 (61.01)	87.5 (72.07)	87.3 (69.14)	53.3 (46.89)	70.3 (58.07)
3	11	95.2 (77.55)	72.5 (58.38)	83.85 (67.97)	64.6 (53.49)	44.6 (41.89)	54.6 (47.69)
4	17	92.7 (74.44)	67.6 (55.32)	80.15 (64.88)	49.0 (44.43)	41.4 (40.04)	45.2 (42.24)
5	20	88.5 (70.22)	63.5 (52.84)	76.0 (61.53)	23.9 (29.25)	20.5 (26.89)	22.2 (28.07)
6	23	80.0 (63.45)	56.0 (48.45)	68.0 (55.95)	20.0 (26.56)	17.0 (24.34)	18.5 (25.44)
7	26	70.0 (56.80)	49.0 (44.43)	59.5 (50.61)	16.0 (23.57)	13.0 (21.12)	14.5 (22.34)
8	31	50.5 (45.29)	45.0 (42.13)	47.75 (43.71)	11.0 (19.34)	8.0 (16.39)	9.5 (17.86)
	Mean	84.3 (69.52)	63.51 (53.08)		45.00 (41.96)	31.65 (33.21)	
Genotype							
SEM±		0.118 (0.178)			0.186 (0.130)		
CD at 5%		0.52 (0.49)			0.52 (0.36)		
CV%		2.3 (2.6)			4.3 (3.1)		
Leaf number							
SEM±		0.376 (0.356)			0.372 (0.2599)		
CD at 5%		1.04 (0.99)			1.03 (0.72)		
CV%		2.3 (2.6)			4.3 (3.1)		
Genotype x leaf no.ö							
SEM±		0.532 (0.504)			0.526 (0.3616)		
CD at 5%		1.47 (1.39)			1.46 (1.02)		
CV%		2.3 (2.6)			4.3 (3.1)		

Figures in parenthesis are arscin transformed values
* Mean of 10 leaf samples
DAS = Days after sowing; S = Susceptible genotype; R = Resistant genotype.

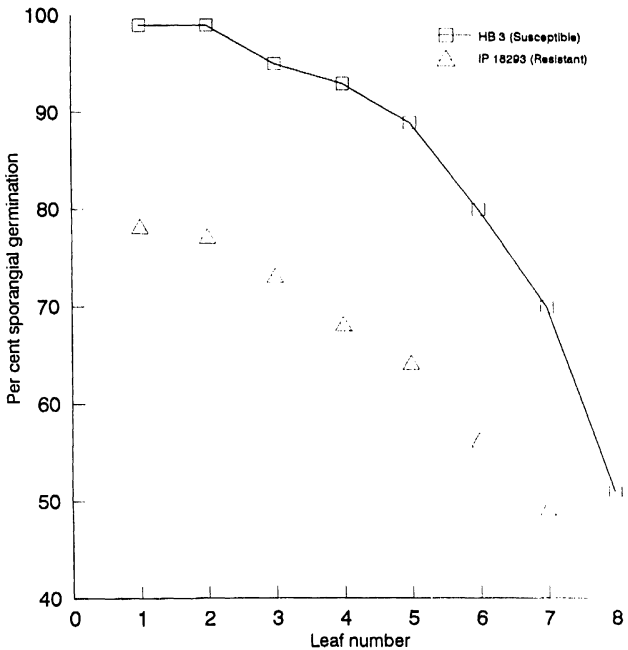

Fig. 1 : Germination of sporangia of *Sclerospora graminicola* on 1st to 8th leaf of a susceptible and a resistant genotype.

The R^2 values were high enough to indicate that the relationship between sporangial germination and leaf age was quadratic. The correlation between sporangial germination and the leaf age was calculated and the correlation coefficients were highly negative (HB 3, -0.9197**; IP 18293 - 0.9883**) implying that the sporangial germination decreases as leaf age increases.

However, the germination of zoospores, studied on the same leaf numbers of the same genotypes, was more drastically reduced. Zoospore germination was reduced from 88.2 per cent to 11.0 per cent on susceptible genotype and from 55.4 to 8.0 per cent on resistant genotype. Whereas sporangial germination was reduced from 100 to 50.5 per cent on susceptible genotype, and from 78 to 45 per cent on resistant genotype (Table 3 and Fig. 2). To know the relationship between zoospore germination and the leaf age, the zoospore germination was plotted against leaf age. The relationship was found to be linear. The regression equations are given below:

ZG 1 = 101.56 - 12.57 X (R^2 = 0.92)

ZG 2 = 65.78 - 7.58 X (R^2 = 0.94)

ZG 1 = Per cent zoospore germination on HB 3

ZG 2 = Per cent zoospore germination on IP 18293

X = leaf age

R^2 = regression coefficient

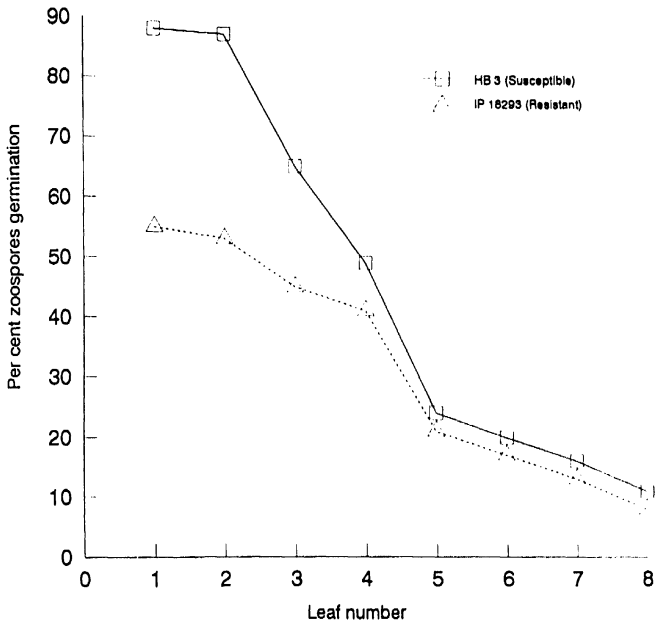

Fig. 2 : Germination of zoospores of *Sclerospora graminicola* on 1st to 8th leaf of a susceptible and a resistant genotype.

R^2 values were high enough to indicate that the relationship between zoospore germination and the leaf age was linear. The correlation between zoospore germination and leaf age was calculated and the correlation coefficients were found to be negative (HB 3, -0.9652**; IP 18293, -0.9748**) implying that the zoospore germination decreases with the increase in leaf age.

4.5 Penetration of the second leaf of resistant and susceptible genotypes by germ tubes of zoospores

The two susceptible genotypes (HB 3 and 7042 S) had significantly more penetrations in all the three durations (HB 3, 47 per cent, 53.6%, 56%; 7042 S, 44.1%, 50.6%, 54.0% at 2, 4, and 6 HAI respectively) than the resistant genotypes (IP 18292, 25.3%, 33.1%, 35.7%; IP 18293 26.5%, 34%, 36.2%; IP 18294, 28%, 37.6%, 39.7%; IP 18296, 31%, 40%, 42%, at 2, 4, and 6 HAI, respectively). However, in all the genotypes, more than 90 per cent penetrations occurred through the epidermis. The per cent penetration significantly increased from 2 HAI to 6 HAI, in both susceptible and resistant genotypes (Table 4).

4.6 Penetration of 1st to 8th leaf of a susceptible and a resistant genotype by zoospore germ tubes

The penetration was highest on the first leaf of the two genotypes, HB 3 and IP 18293, decreased linearly on the subsequent leaves, and was the lowest on

Table 4: Penetration of the second leaf of resistant and susceptible genotypes by germ tubes of zoospores of *S. graminicola*.

Genotype	2 h after inoculation*			4 h after inoculation*			6 h after inoculation*			Mean		
	PE(%)	PS(%)	TP(%)	PE(%)	PS(%)	TP(%)	PE(%)	PS(%)	TP(%)	PE(%)	PS(%)	TP(%)
HB 3(S)	46.5 (42.99)	0.5 (4.05)	47.0 (43.28)	53.1 (46.78)	0.5 (4.05)	53.6 (47.07)	54.9 (47.81)	1.1 (5.94)	56.0 (48.45)	51.5 (45.86)	0.7 (4.68)	52.2 (46.26)
7042 S(S)	43.4 (41.21)	0.7 (4.73)	44.1 (41.61)	49.9 (44.94)	0.7 (4.73)	50.6 (45.34)	53.2 (46.84)	0.8 (5.07)	54.0 (47.29)	48.83 (44.33)	0.73 (4.84)	49.57 (44.75)
IP 18292(R)	24.8 (29.86)	0.5 (4.05)	25.3 (30.19)	32.3 (34.63)	0.8 (5.07)	33.1 (35.12)	34.3 (35.85)	1.4 (6.74)	35.7 (36.69)	30.47 (33.44)	0.9 (5.28)	31.37 (33.99)
IP 18293(R)	25.2 (30.12)	1.3 (6.48)	26.5 (30.97)	32.5 (34.75)	1.5 (6.94)	34.0 (35.66)	34.4 (35.91)	1.8 (7.65)	36.2 (36.99)	30.7 (33.59)	1.53 (7.02)	32.23 (34.54)
IP 18294(R)	27.5 (31.62)	0.5 (4.05)	28.0 (31.94)	36.9 (37.4)	0.7 (4.73)	37.6 (37.82)	38.2 (38.17)	1.5 (6.94)	39.7 (39.05)	34.2 (35.73)	0.9 (5.24)	35.10 (36.27)
IP 18296(R)	30.4 (33.45)	0.6 (4.39)	31.0 (33.82)	38.6 (38.41)	1.4 (6.69)	40.0 (39.23)	40.6 (39.58)	1.4 (6.69)	42.0 (40.39)	36.53 (37.15)	1.13 (5.93)	37.67 (37.82)
Mean	32.97 (34.88)	0.68 (4.63)	33.65 (35.30)	40.55 (39.49)	0.93 (5.34)	41.48 (40.04)	42.60 (40.69)	1.33 (6.50)	43.93 (41.48)			

		SEM±	CD at 5%
PE	Genotype	0.287 (0.173)	0.79 (0.48)
	HAI	0.203 (0.122)	0.56 (0.34)
	Genotype x HAI	0.497 (0.299)	1.38 (0.83)
PS	Genotype	0.063 (0.165)	0.17 (0.46)
	HAI	0.045 (0.117)	0.12 (0.32)
	Genotype x HAI	0.109 (0.286)	0.30 (0.79)
TP	Genotype	0.287 (0.172)	0.79 (0.48)
	HAI	0.203 (0.122)	0.56 (0.34)
	Genotype x HAI	0.497 (0.298)	1.38 (0.83)

CV% PE 4.1 (2.5)
PS 35.1 (16.4)
TP 4.0 (2.4)

Figures in parenthesis are arcsin transformed values
* Mean of 10 leaf samples
PE = Penetration through epidermis; PS = Penetration through stomata; TP = Total penetration;
HAI = Hours after inoculation; S = Susceptible genotype; R = Resistant genotype

the 8th leaf (from 57% to 8% in HB 3 and from 37.2% to 4% in IP 18293) (Table 5 and Fig. 3). The per cent penetration was plotted against leaf age and the relationship was found to be linear with the following regression equations:

P1 = 67.55 - 7.720 X (R^2 = 0.96)

P2 = 44.75 - 5.017 X (R^2 = 0.98)

P1 = Per cent penetration on HB 3

P2 = Per cent penetration on IP 18293

X = leaf age

R^2 = regression coefficient

The R^2 values were high enough to indicate that the relationship between penetration and the leaf age was linear. The correlation coefficients between penetration and leaf age were negative (HB 3, -0.9837**; IP 18293, -0.9917**) implying that penetration decreases with the increase in leaf age.

4.7 Effect of temperature on *in vivo* germination of sporangia and zoospores and leaf penetration by germ tubes.

The results showed that the sporangial germination, on the leaf surface of HB 3 (susceptible) and IP 18293 (resistant), was highest at 20° and 25°C (HB 3, 98.5 & 98.3%; IP 18293, 76.5 & 76.3%), and decreased with the further increase or decrease in temperature (Table 6 and Fig. 4). The lowest germination occurred at 35°C (HB 3, 50.2%; IP 18293, 28.1%). Similarly, the zoospore germination was also

Table 5: Penetration of 1st to 8th leaf of a susceptible and a resistant genotype by the zoospore germ tubes.

		Per cent penetration*								
		HB 3(S)			IP 18293(R)			Mean		
Leaf No.	DAS	PE	PS	TP	PE	PS	TP	PE	PS	TP
1	5	56.0	1.0	57.0	35.0	2.0	37.2	45.6	1.5	47.1
		(48.45)	(5.64)	(49.03)	(36.39)	(8.11)	(37.58)	(42.42)	(6.87)	(43.30)
2	7	54.9	1.1	56.0	34.4	1.8	36.2	44.65	1.45	46.1
		(47.81)	(5.94)	(48.45)	(35.91)	(7.65)	(36.99)	(41.86)	(6.79)	(42.72)
3	11	44.0	1.0	45.0	31.0	1.0	32.0	37.5	1.0	38.5
		(41.55)	(5.64)	(42.13)	(33.83)	(5.64)	(34.44)	(37.69)	(5.64)	(38.29)
4	17	39.5	1.0	40.5	24.0	1.0	25.0	31.75	1.0	32.75
		(38.94)	(5.64)	(39.52)	(29.33)	(5.64)	(29.99)	(34.13)	(5.64)	(34.76)
5	20	22.0	1.0	23.0	17.2	0.8	18.0	19.6	0.9	20.5
		(27.96)	(5.64)	(28.65)	(24.49)	(5.07)	(25.09)	(26.23)	(5.35)	(26.87)
6	23	18.2	0.8	19.0	14.5	0.5	15.0	16.35	0.65	17.0
		(25.24)	(5.07)	(25.83)	(22.37)	(4.05)	(22.77)	(23.81)	(4.56)	(24.30)
7	26	13.5	0.5	14.0	9.5	0.5	10.0	11.5	0.5	12.00
		(21.54)	(4.05)	(21.95)	(17.92)	(4.05)	(18.40)	(19.73)	(4.05)	(20.18)
8	31	7.5	0.5	8.0	3.5	0.5	4.0	5.5	0.5	6.00
		(15.86)	(4.05)	(16.39)	(10.69)	(4.05)	(11.45)	(13.27)	(4.05)	(13.92)
	Mean	31.95	0.86	32.81	21.16	1.01	22.18			
		(33.42)	(5.21)	(33.99)	(26.36)	(5.53)	(27.09)			

		SEM±	CD at 5%	CV%
PE	Genotype	0.143	0.39	4.8
		(0.167)	(0.31)	(3.3)
	Leaf number	0.286	0.79	4.8
		(0.2202)	(0.61)	(3.3)
	Genotype x	0.404	1.12	4.8
	leaf number	(0.3114)	(0.86)	(3.3)
PS	Genotype	0.0353	0.098	33.7
		(0.0965)	(0.27)	(16.1)
	Leaf number	0.0706	0.196	33.7
		(0.193)	(0.53)	(16.1)
	Genotype x	0.0999	0.277	33.7
	leaf number	(0.273)	(0.76)	(16.1)
TP	Genotype	(0.154)	0.43	5.0
		(0.1148)	(0.32)	(3.4)
	Leaf number	0.308	0.85	5.0
		(0.2295)	(0.64)	(3.4)
	Genotype x	0.436	1.21	5.0
	leaf number	(0.3246)	(0.89)	(3.4)

Figures in parenthesis are arcsin transformed values; * = Mean of 10 leaf samples; DAS = Days after sowing; PE = Penetration through epidermis; PS = Penetration through stomata; TP = Total penetration; S = Susceptible genotype; R = Resistant genotype.

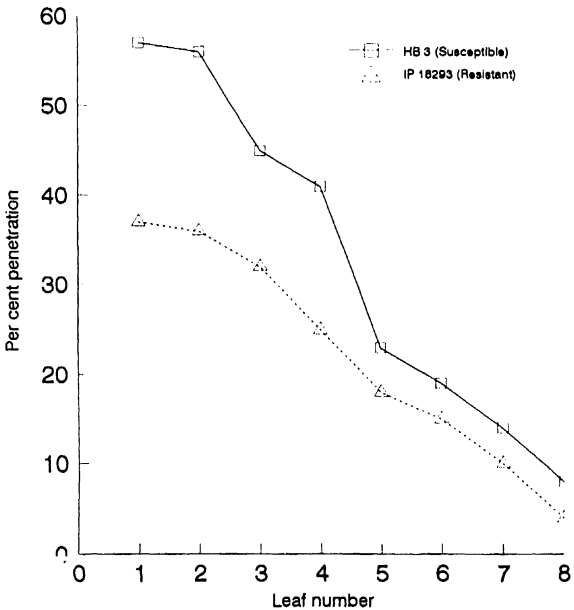

Fig. 3: Penetration of 1st to 8th leaf of a susceptible and a resistant genotype by germ tubes of zoospores of *Sclerospora graminicola.*

Table 6: Effect of temperature on *in vivo* germination of sporangia and zoospores and leaf penetration by the germ tubes.

Temperature	Genotype	Per cent sporangial germination*	Per cent zoospore germination*	Per cent penetration*
15°C	HB 3(S)	80.00 (63.45)	42.60 (40.74)	13.90 (21.88)
	IP 18293(R)	58.00 (49.60)	18.60 (25.54)	8.20 (16.61)
20°C	HB 3(S)	98.50 (83.12)	87.30 (69.14)	56.00 (48.45)
	IP 18293(R)	76.50 (61.01)	53.30 (46.89)	36.20 (36.99)
25°C	HB 3(S)	98.30 (82.72)	68.60 (55.92)	43.00 (40.98)
	IP 18293(R)	76.30 (60.87)	34.60 (36.03)	28.00 (31.94)
30°C	HB 3(S)	96.20 (78.92)	63.70 (52.95)	37.50 (37.76)
	IP 18293(R)	74.10 (59.41)	29.50 (32.89)	22.50 (28.31)
35°C	HB 3(S)	50.20 (45.11)	9.30 (17.74)	9.40 (17.83)
	IP 18293(R)	28.10 (32.01)	4.40 (12.04)	4.00 (11.48)
SEM±		0.374 (0.385)	0.370 (0.2772)	0.364 (0.2816)
C.D. at 5%		1.04 (1.07)	1.03 (0.77)	1.01 (0.78)
CV%		1.6 (2.0)	2.8 (2.2)	4.5 (3.0)

Figures in parenthesis are arscin transfomed values
* = Mean of 10 leaf samples
S = Susceptible genotype; R = Resistant genotype

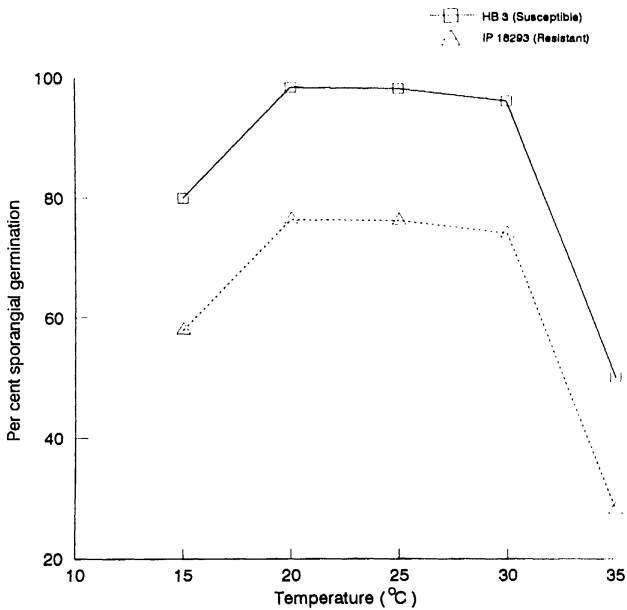

Fig 4: Effect of temperature on *in vivo germination of sporangia of Sclerospora graminicola.*

highest at 20°C and decreased with the decrease or increase in temperature, being the lowest at 35°C on the leaf surfaces of both the genotypes (Table 6 and Fig. 5).

Penetration by the germ tubes was also highest at 20°C and decreased with the decrease or increase in temperature, being the lowest at 35°C on the leaf surfaces of both the genotypes (Table 6 and Fig. 6).

The regression analysis revealed that the optimum temperature for sporangial and zoospore germination was 23.3°C for both the genotypes. The optimum temperature for penetration was 24.3°C and 24.1°C for HB 3 and IP 18293, respectively (Table 7). The regression equations are given below:

SG 1 = $-99.50 + 17.462 X - 0.374 X^2$ ($R^2 = 0.928$)

SG 2 = $-121.50 + 17.470 X - 0.374 X^2$ ($R^2 = 0.929$)

ZG 1 = $-203.50 + 24.54 X - 0.562 X^2$ ($R^2 = 0.910$)

ZG 2 = $-120.00 + 14.10 X - 0.303 X^2$ ($R^2 = 0.790$)

P 1 = $-172.60 + 18.44 X - 0.379 X^2$ ($R^2 = 0.845$)

P 2 = $-117.52 + 12.46 X - 0.258 X^2$ ($R^2 = 0.854$)

SG 1 = Per cent sporangial germination on HB 3

SG 2 = Per cent sporangial germination on IP 18293

ZG 1 = Per cent zoospore germination on HB 3

ZG 2 = Per cent zoospore germination on IP 18293

P 1 = Per cent penetration on HB 3

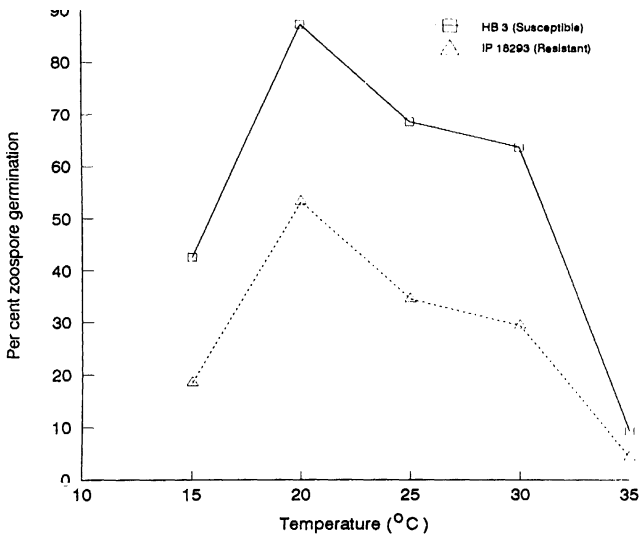

Fig 5: Effect of temperature on *in vivo* germination of zoospores of *Sclerospora graminicola*.

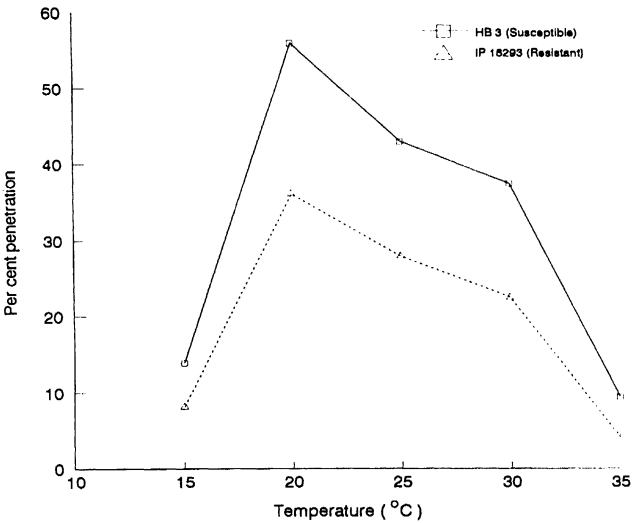

Fig. 6 : Effect of temperature on leaf penetration by germ tubes of zoospores of *Sclerospora graminicola.*

P 2 = Per cent penetration on IP 18293

X = Temperature

R^2 = Regression coefficient

Table 7: Optimum temperature for sporangial and zoospore germination and germ tube penetration.

	Optimum temperature (°C)		
Genotype	Sporangial germination	Zoospore germination	Penetration
HB 3(S)	23.3	23.3	24.3
IP 18293(R)	23.3	23.3	24.1

S = Susceptible genotype; R = Resistant genotype

4.8 Colonization of 1st to 5th leaf by *Sclerospora graminicola* in resistant and susceptible genotypes

Colonization of *S. graminicola* was not observed in the leaf tissues of the resistant genotypes IP 18292 and IP 18293 at all stages of growth. In resistant genotype, IP 18294, none of the 1st leaf samples were colonized, but colonization did occur in 10-20 per cent leaf samples from the higher growth stages. In resistant genotype IP 18296, colonization was observed in 10-20 per cent leaf samples at all stages of plant growth. However, in both the cases, the growth of the mycelium remained restricted and there was no haustorial formation. In susceptible genotypes HB 3 and 7042 S, all the leaf samples from all the growth stages showed colonization (Table 8). In general, in susceptible genotypes, the mycelial growth

Table 8: Colonization of leaf tissues of resistant and susceptible genotypes by *S. graminicola* at different stages of plant growth.

Leaf No.	DAS	Genotype	Leaf samples showing mycelium (%)	Remarks
1	5	HB 3 (S)	100	Mycelium was found throughout the leaves
		7042 S(S)	100	--do--
		IP 18292(R)	0	No Mycelium
		IP 18293(R)	0	--do--
		IP 18294(R)	0	--do--
		IP 18296(R)	20	Only a few hyphal bits with extremely small length.
2	7	HB 3(S)	100	Mycelium was found throughout the leaves
		7042 S(S)	100	--do--
		IP 18292(R)	0	No mycelium
		IP 18293(R)	0	--do--
		IP 18294(R)	20	Only a few hyphal bits with extremely small length.
		IP 18296(R)	20	--do--
3	11	HB 3(S)	100	Mycelium was found throughout of the leaves.
		7042 S(S)	100	--do--
		IP 18292(R)	0	No mycelium
		IP 18293(R)	0	--do--
		IP 18294(R)	20	Only a few hyphal bits with extremely small length.
		IP 18296(R)	20	--do--
4	17	HB 3(S)	100	Mycelium was found throughout the leaves.
		7042 S(S)	100	--do--
		IP 18292(R)	0	No mycelium
		IP 18293(R)	0	No mycelium
		IP 18294(R)	10	Only a few hyphal bits with extremely small length.
		IP 18296(R)	10	--do--
5	20	HB 3(S)	100	Mycelium was found throughout the leaves.
		7042 S(S)	100	--do--
		IP 18292(R)	0	No mycelium
		IP 18293(R)	0	--do--
		IP 18294(R)	10	Only a few hyphal bits with extremely small length.
		IP 18296(R)	10	--do--

DAS = Days after sowing; R = Resistant genotype; S = Susceptible genotype.

increased with increase in number of days after inoculation (DAI), and the leaf tissues were colonized completely, 5 DAI and the fungus produced haustoria.

4.9 Anatomical basis of resistance/susceptibility to *S. graminicola*.

4.9.1 Thickness of cuticle on upper and lower surfaces of leaves of resistant and susceptible genotypes at various stages of plant growth.

Thickness of cuticle did not vary significantly between upper and lower surfaces of the same leaf, in all the leaves and for all the genotypes except on 5th leaf of IP 18292, where the thickness of cuticle was significantly more on the upper surface of the leaf than on the lower surface (Table 9).

However, cuticle thickness on the leaves of resistant genotypes (both on upper and lower surfaces) was significantly more than the susceptible ones (P=0.05) in many cases. Among the resistant genotypes, IP 18293 showed the highest mean cuticle thickness and IP 18296 the least. However, there were exceptions where no significant differences were observed between susceptible and resistant genotypes (Table 9).

Table 9: Thickness of cuticle on upper and lower surfaces of 1st to 8th leaf of four resistant and two susceptible genotypes.

Leaf No.	DAS	Cuticle thicknes on upper surface of the leaf (µm)							Cuticle thickness on lower surface of the leaf (µm)						
		HB 3(S)	7042 S(S)	IP 18292(R)	IP 18293(R)	IP 18294(R)	IP 18296(R)	Mean	HB3 (S)	7042 S(S)	IP 18292(R)	IP 18293(R)	IP 18294(R)	IP 18296(R)	Mean
1	4	1.25	1.18	1.31	1.63	1.60	1.31	1.38	1.22	1.17	1.31	1.63	1.60	1.31	1.37
2	7	1.57	1.63	1.89	1.94	1.86	1.92	1.80	1.57	1.63	1.89	1.94	1.86	1.92	1.80
3	11	2.14	2.11	2.18	2.19	2.18	2.21	2.17	2.14	2.06	2.14	2.16	2.14	2.14	2.13
4	17	2.40	2.45	2.62	2.66	2.53	2.49	2.53	2.37	2.42	2.59	2.64	2.53	2.48	2.50
5	20	2.80	2.85	3.04	3.04	2.88	2.85	2.91	2.78	2.85	2.91	2.98	2.85	2.85	2.87
6	23	3.10	3.17	3.36	3.39	3.26	3.23	3.25	3.07	3.17	3.29	3.36	3.23	3.23	3.23
7	26	3.46	3.52	3.74	3.78	3.68	3.62	3.63	3.46	3.49	3.74	3.74	3.65	3.58	3.61
8	31	3.84	3.87	4.16	4.19	4.00	3.97	4.01	3.81	3.84	4.13	4.16	3.97	3.94	3.97
	Mean	2.57	2.59	2.79	2.85	2.75	2.70		2.55	2.58	2.75	2.83	2.73	2.68	

	SEM ±	CD at 5%
Genotype x Leaf surface	0.015	0.04
Leaf No. x Leaf surface	0.017	0.05
Genotype x Leaf No. x Leaf surface	0.042	0.12
CV %	7.0	

DAS = Days after sowing; S = Susceptible genotype; R = Resistant genotype.

The cuticle thickness was the least in the 1st leaf, and there was linear increase in the thickness of cuticle from the 1st leaf to 8th leaf, on both the surfaces of all the genotypes (Fig. 7 and 8).

The relationship between cuticle thickness and leaf age was linear and the regression equations are given below for different genotypes.

CT 1 U = 0.9168 + 0.3674 X (R^2= 0.995)

CT 1 L = 0.9036 + 0.3664 X (R^2 = 0.994)

CT 2 U = 0.8907 + 0.3793 X (R^2 = 0.995)

CT 2 L = 0.8779 + 0.3779 X (R^2 = 0.996)

CT 3 U = 1.0111 + 0.3947 X (R^2 = 0.996)

CT 3 L = 0.9950 + 0.3900 X (R^2 = 0.996)

CT 4 U = 1.1864 + 0.3702 X (R^2 = 0.996)

CT 4 L = 1.1843 + 0.3649 X (R^2 = 0.995)

CT 5 U = 1.1689 + 0.3511 X (R^2 = 0.996)

CT 5 L = 1.1682 + 0.3468 X (R^2 = 0.996)

CT 6 U = 1.0639 + 0.3636 X (R^2 = 0.993)

CT 6 L = 1.0554 + 0.3613 X (R^2 = 0.993)

CT 1 U = cuticle thickness on upper surface of HB 3

CT 1 L = cuticle thickness on lower surface of HB 3

CT 2 U = cuticle thickness on upper surface of 7042 S

CT 2 L = cuticle thickness on lower surface of 7042 S

CT 3 U = cuticle thickness on upper surface of IP 18292

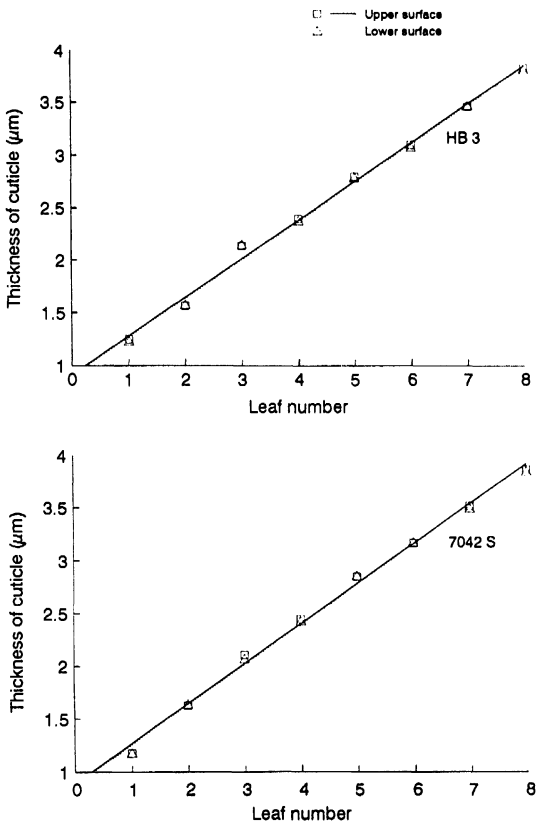

Fig. 7 : Thickness of cuticle on upper and lower surfaces of 1st to 8th leaf of two susceptible genotypes (HB 3 and 7042 S).

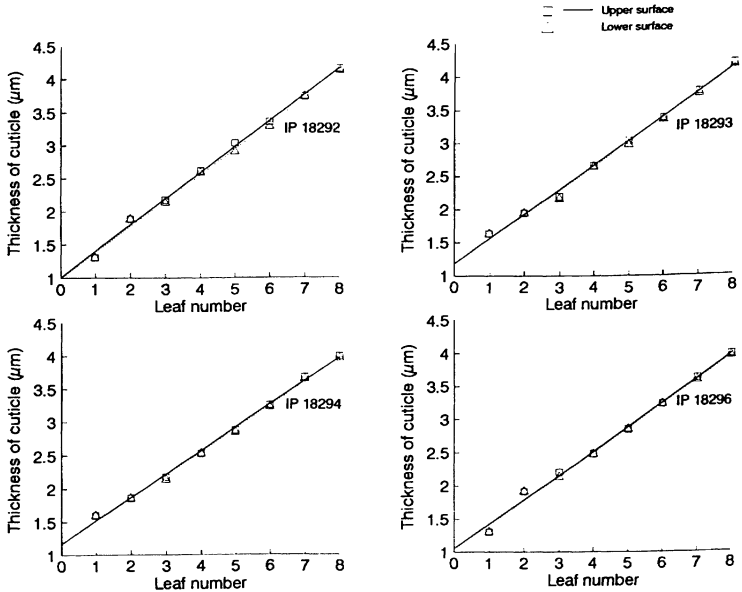

Fig. 8 : Thickness of cuticle on upper and lower surfaces of 1st to 8th leaf of four resistant genotypes (IP 18292, IP 18293, IP 18294 and IP 18296).

CT 3 L = cuticle thickness on lower surface of IP 18292

CT 4 U = cuticle thickness on upper surface of IP 18293

CT 4 L = cuticle thickness on lower surface of IP 18293

CT 5 U = cuticle thickness on upper surface of IP 18294

CT 5 L = cuticle thickness on lower surface of IP 18294

CT 6 U = cuticle thickness on upper surface of IP 18296

CT 6 L = cuticle thickness on lower surface of IP 18296

X = Leaf age

R^2 = Regression coefficient

The R^2 values were high and indicated that the relationship between cuticle thickness and leaf age was linear and the correlations between cuticle thickness and leaf age were positive (Table 10) implying that as the leaf age increases, the cuticle thickness also increases.

Table 10: Correlation coefficients (r) between thickness of cuticle on upper and lower surfaces of the leaf and leaf age in resistant and susceptible genotypes.

Genotype	Upper surface of the leaf	Lower surface of the leaf
HB 3(S)	+ 0.9977**	+ 0.9974**
7042 S(S)	+ 0.9980**	+ 0.9981**
IP 18292(R)	+ 0.9982**	+ 0.9981**
IP 18293(R)	+ 0.9983**	+ 0.9977**
IP 18294(R)	+ 0.9985**	+ 0.9981**
IP 18296(R)	+ 0.9968**	+ 0.9971**

** Significant at 1%
S = Susceptible genotype; R = Resistant genotype

4.9.2 Amount of wax in leaves of resistant and susceptible genotypes at various stages of plant growth

IP 18292, the most resistant genotype, showed the least wax of all the genotypes tested. Among other genotypes, the three resistant genotypes (IP 18293, IP 18294, IP 18296) possessed more wax, in some cases significantly more, than the two susceptible genotypes (HB 3 and 7042 S) (Table 11).

The results (Fig. 9) also show that the amount of wax significantly increased from 1st to 7th leaf in HB 3, 7042 S, IP 18293, IP 18294, and IP 18296. However, there are no significant differences between 7th and 8th leaf in all the genotypes tested. In case of IP 18292, the wax content did not increase significantly from one leaf to the next leaf stage, but the wax content increased significantly in alternate leaves.

A linear regression between amount of wax and the leaf age was calculated and the regression equations are given below for different genotypes.

W 1 = 0.8689 + 0.2744 X (R^2 = 0.973)

W 2 = 0.9438 + 0.2763 X (R^2 = 0.971)

W 3 = 0.3454 + 0.0651 X (R^2 = 0.983)

W 4 = 1.1775 + 0.2713 X (R^2 = 0.980)

W 5 = 1.1154 + 0.2742 X (R^2 = 0.981)

W 6 = 1.0479 + 0.2784 X (R^2 = 0.980)

W 1 = Amount of wax in HB 3

W 2 = Amount of wax in 7042 S

Table 11:Amount of wax in 1st to 8th leaf of four resistant and two susceptible genotypes.

Leaf No.	DAS	Amount of wax (mg g^{-1} fresh weight)						
		HB 3(S)	7042 S(S)	IP 18292(R)	IP 18293(R)	IP 18294(R)	IP 18296(R)	Mean
1	5	1.17	1.22	0.40	1.52	1.47	1.39	1.19
2	7	1.27	1.39	0.47	1.65	1.60	1.52	1.31
3	11	1.75	1.82	0.55	1.94	1.87	1.85	1.63
4	17	2.05	2.12	0.60	2.25	2.18	2.15	1.89
5	20	2.20	2.27	0.69	2.52	2.50	2.44	2.10
6	23	2.60	2.79	0.77	2.92	2.87	2.85	2.46
7	26	2.89	2.92	0.80	3.19	3.14	3.09	2.67
8	31	2.92	3.00	0.84	3.22	3.19	3.14	2.71
	Mean	2.10	2.19	0.64	2.39	2.35	2.30	

	SEM±	CD at 5%
Genotype	0.013	0.04
Leaf No.	0.015	0.04
Genotype x Leaf No.	0.037	0.10
CV %	2.6	

DAS = Days after sowing; S = Susceptible genotype; R = Resistant genotype

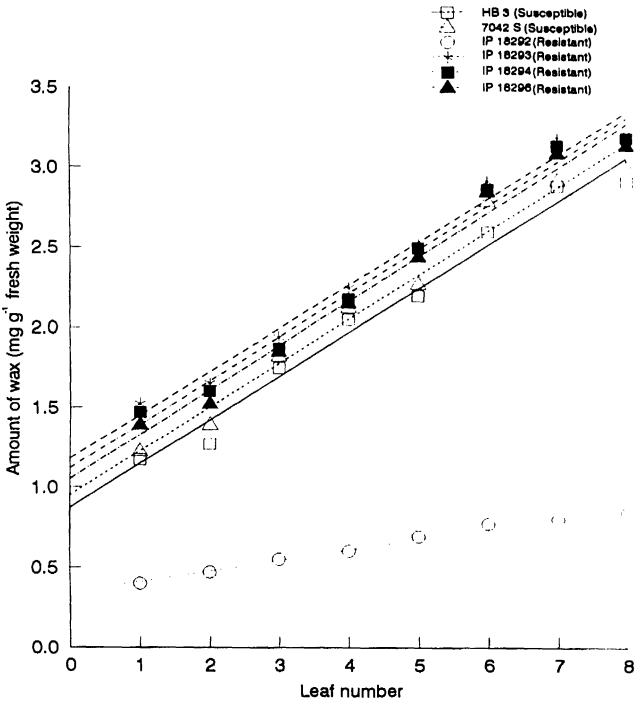

Fig. 9: Amount of wax in 1st to 8th leaf of two susceptible and four resistant genotypes.

W 3 = Amount of wax in IP 18292

W 4 = Amount of wax in IP 18293

W 5 = Amount of wax in IP 18294

W 6 = Amount of wax in IP 18296

X = Leaf age

R = Regression coefficient

R^2 values were high enough to indicate that the relationship between wax content and leaf age was linear and the correlations were positive (Table 12) implying that as the leaf age increases the wax content also increases.

Table 12: Correlation coefficients (r) between amount of wax and leaf age in resistant and susceptible genotypes.

Genotype	Correlation coefficients
HB 3(S)	+ 0.9885**
7042 S (S)	+ 0.9876**
IP 18292 (R)	+ 0.9926**
IP 18293 (R)	+ 0.9913**
IP 18294 (R)	+ 0.9918**
IP 18296 (R)	+ 0.9913**

** Significant at 1%
S = Susceptible genotype; R = Resistant genotype

4.9.3 Number of stomata on upper and lower surface of the leaves of resistant and susceptible genotypes

Number of stomata did not vary significantly between upper and lower surfaces of leaves in resistant and also susceptible genotypes. From the data it is clear that the number of stomata did not differ significantly between the

susceptible (HB 3 and 7042 S) and resistant (IP 18294 and IP 18296) genotypes also. However, two susceptible genotypes (HB 3 & 7042 S) had significantly fewer stomata on the lower surface as compared to the resistant genotypes IP 18292 and IP 18293. It was also observed that the number of stomata did not differ significantly between susceptible genotype HB 3 and the resistant genotype IP 18292 on the upper surface of the leaf (Table 13).

Table 13: Number of stomata on the leaf surface of resistant and susceptible genotypes.

	Number of stomata per mm^2		
Genotype	Upper surface	Lower surface	Mean
HB 3(S)	55.49	58.07	56.78
7042 S(S)	52.34	52.82	52.58
IP 18292(R)	58.93	63.70	61.31
IP 18293(R)	69.33	70.09	69.71
IP 18294(R)	52.44	52.65	52.55
IP 18296(R)	55.53	56.72	56.13
Mean	57.34	59.01	
	SEM ±	CD at 5%	
Genotype	1.391	3.86	
Leaf surface	0.803	2.23	
Genotype x leaf surface	1.967	5.45	
CV %	10.7		

* Mean of 10 leaf samples
S = Susceptible genotype; R = Resistant genotype.

4.10 Biochemical basis of resistance/susceptibility to the disease

4.10.1 Phenols

4.10.1.1 Total phenol content in resistant and susceptible genotypes at different stages of plant growth

The total phenols were significantly more in resistant genotypes than the susceptible genotypes at 7 and 11 days after sowing (DAS). However, the total phenols did not vary significantly between resistant genotypes IP 18292, IP 18293, and IP 18296 and the susceptible genotype HB 3, 5 DAS.

The total phenols increased with the increase in the leaf age of the seedlings in all the genotypes, irrespective of their resistance/susceptibility (Table 14).

4.10.1.2 Total phenol content in inoculated and non-inoculated leaves of resistant and susceptible genotypes

The total phenols were significantly more in non-inoculated leaves of resistant genotypes than in susceptible ones. The total phenols were also significantly more in resistant genotypes than in susceptible ones in inoculated leaves and there was an increase in total phenols content after inoculation in both genotypes. The total phenol content was highest in the resistant genotype IP 18292, than all other genotypes (Table 15).

Table 14: Total phenols in resistant and susceptible genotypes at different stages of plant growth.

	Total phenols (%)						
DAS	HB 3(S)	7042 S(S)	IP 18292(R)	IP 18293(R)	IP 18294(R)	IP 18296(R)	Mean
5	1.03	0.86	1.11	1.04	1.13	1.07	1.04
7	1.91	1.83	2.09	2.25	2.09	2.21	2.06
11	2.71	2.37	3.13	3.38	3.16	3.32	3.01
Mean	1.88	1.69	2.11	2.23	2.13	2.19	

	SEM±	CD at 5%
Genotype	0.019	0.06
DAS	0.014	0.04
Genotype × DAS	0.034	0.09
CV %	2.3	

DAS = Days after sowing; S = Susceptible genotype; R = Resistant genotype

Table 15: Total phenols in inoculated and non-inoculated leaves of susceptible and resistant genotypes.

Genotype	Total phenols (%)		Mean
	Non-inoculated	Inoculated	
HB 3(S)	1.59	1.67	1.63
7042 S(S)	1.48	1.55	1.52
IP 18292(R)	1.92	2.04	1.98
IP 18293(R)	1.86	1.98	1.92
IP 18294(R)	1.75	1.87	1.81
IP 18296(R)	1.81	1.93	1.87
Mean	1.74	1.84	
	SEM±	CD at 5%	
Genotype	0.006	0.02	
I/NI	0.004	0.01	
Genotype × I/NI	0.009	0.03	
CV %	0.7		

S = Susceptible genotype; R = Resistant genotype; I = Inoculated; NI = Non-inoculated.

4.10.1.3 Qualitative analysis of phenols

Preliminary investigations were carried out to identify the phenolic compounds in disease free leaves of susceptible (HB 3 and 7042 S) and resistant (IP 18292 and IP 18293) genotypes, in 1st leaf stage. The sample extracts were analyzed for their total phenols (on tannic acid standard) using Folin-Ciocalteu's method and the extracts equivalent to 20 µg phenols were spotted on TLC plates. A good separation of phenols was obtained using n butanol : acetic acid : water (4:1:5) system and silver nitrate spray reagent in acetone. In case of resistant genotypes (IP 18292 and IP 18293) a distinct band was identified (phenol unknown) close to the solvent front which is absent in susceptible genotypes (HB 3 and 7042 S) (Plate 12).

4.10.2 Total soluble sugars

4.10.2.1 Total soluble sugars in resistant and susceptible genotypes at different stages of plant growth

The data (Table 16) shows that the total soluble sugars (TSS) were significantly more in the susceptible genotype, 7042 S than all the resistant genotypes tested, at all stages of plant growth. However, the TSS did not differ significantly between susceptible genotype 7042 S and resistant genotype IP 18293, 5 DAS.

TSS were also significantly more in susceptible genotype HB 3, than in resistant genotype IP 18296, 5 DAS; IP 18294 and IP 18296, 7 DAS; and in all the 4

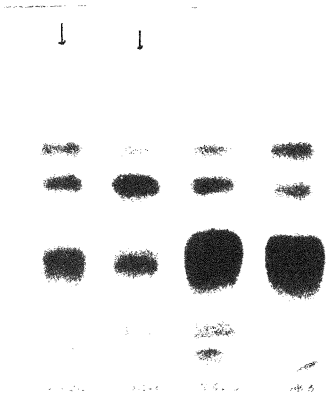

Plate 12: Separation of total phenols by Thin layer chromatography.
IP 18292 and IP 18293 (Resistant); 7042 S and HB 3 (Susceptible)

Table 16: Total soluble sugars in resistant and susceptible genotypes at different stages of plant growth.

	Total soluble sugars (%)						
DAS	HB 3(S)	7042 S(S)	IP 18292(R)	IP 18293(R)	IP 18294(R)	IP 18296(R)	Mean
5	1.49	1.51	1.46	1.47	1.46	1.44	1.47
7	1.59	1.63	1.58	1.58	1.54	1.53	1.57
11	1.78	1.83	1.72	1.73	1.70	1.69	1.74
Mean	1.62	1.66	1.59	1.59	1.57	1.55	

	SEM±	CD at 5%
Genotype	0.009	0.03
DAS	0.007	0.02
Genotype × DAS	0.017	0.05
CV %	1.5	

DAS = Days after sowing; S = Susceptible genotype; R = Resistant genotype

resistant genotypes, 11 DAS. However, the TSS did not vary significantly between HB 3 and IP 18292 and IP 18293, 5 and 7 DAS; between HB 3 and IP 18294 5 DAS.

The TSS increased with the increase in the age of the seedlings, in all the genotypes tested.

4.10.2.2 Total soluble sugars (TSS) in inoculated and non-inoculated leaves of resistant and susceptible genotypes

The TSS were significantly more in inoculated leaves of susceptible genotypes (HB 3, 1.66%; 7042 S, 1.67%), than in non-inoculated leaves (HB 3, 1.56%; 7042 S, 1.58%), while in resistant genotypes, TSS did not differ significantly between inoculated and non-inoculated leaves (Table 17).

Table 17: Total soluble sugars in inoculated and non-inoculated leaves of susceptible and resistant genotypes.

Genotype	Total soluble sugars (%)		Mean
	Non-inoculated	Inoculated	
HB 3(S)	1.56	1.66	1.61
7042 S(S)	1.58	1.67	1.63
IP 18292(R)	1.52	1.53	1.53
IP 18293(R)	1.53	1.54	1.53
IP 18294(R)	1.50	1.50	1.50
IP 18296(R)	1.51	1.52	1.52
Mean	1.53	1.57	

	SEM±	CD at 5%
Genotype	0.020	0.06
I/NI	0.012	0.04
Genotype × I/NI	0.028	0.09
CV %	2.6	

S = Susceptible genotype; R = Resistant genotype; I = Inoculated; NI = Non-inoculated.

DISCUSSION

CHAPTER V

DISCUSSION

The role of sporangia in the epidemiology of pearl millet downy mildew incited by *Sclerospora graminicola* has been clearly established in the past (Singh and Williams, 1980; Subramanya *et al.*, 1982). However, the information on basic aspects of the biology and epidemiology of the pathogen is still inadequate. Similarly, the mechanism(s) of disease resistance is yet to be elucidated. Hence, studies on germination of sporangia and zoospores on the leaf surface, penetration by the germ tubes, and colonization in the leaf tissue of susceptible and resistant genotypes were undertaken. Also, studies on anatomical and biochemical differences between susceptible and resistant genotypes were carried out.

In general, the sequence of events taking place from sporangial germination to successful establishment, as observed for this host-parasite system, is in agreement with what has been described for other downy mildew pathogens (Dalmacio and Exconde, 1969-70; Yeh and Frederiksen 1980; Lebeda and Reinink, 1991; Dai *et al.*, 1992; Mouzeyar *et al.*, 1993; Clark and Spencer-Phillips 1994).

It was found that most sporangia germinated indirectly by producing zoospores. Occasionally, direct germination of sporangia by forming a germ tube has also been observed (Plate 4). These observations are in agreement with the

earlier report (Singh, 1995). The encysted zoospores germinate by forming a germ tube (Plate 5). The tip of the germ tube enlarged and formed a globose appressorium (Plate 6). In some cases, the germ tubes penetrated the leaves without forming appressoria (Plate 7).

Fluorescence microscopic studies showed that the germ tubes penetrated the leaf directly through epidermal cells (Table 4 and Plate 8). Rarely, germ tubes enter into the leaf through stomata (Table 4 and Plate 9). These results are in agreement with that of Bhatnagar (1988). This mode of penetration has been considered to be a "known tendency" by Brown and Harvey (1927). Wood (1967) suggested the possibility of wax ducts in cuticle and ectodesmata in outer epidermal cell walls forming passage for fungal penetration. However, this is in contrast to the observation made by Subramanya *et al.* (1983), Mauch-Mani *et al.* (1989), and Shetty (1989) who reported that the natural openings in the form of stomata served as excellent sites for infection.

After penetrating through the epidermal layer, the fungus spread into the intercellular spaces of mesophyll and colonized the leaf tissue inter-and intracellularly. The mycelium was coenocytic (Plate 10) and produced numerous haustoria, which were di-or trichotomously branched (Plate 11). These findings are supported by the observations made by Singh (1974) and Bhatnagar (1988).

Histopathological studies on susceptible and resistant genotypes showed that the germination of sporangia and zoospores, and penetration by germ tubes,

were significantly lower on the leaves of resistant genotypes than the leaves of susceptible ones (Table 2 and 4). Similar observations were reported by Bhatnagar (1988) in pearl millet and in lettuce by Lebeda and Reinink (1991). It appears that exogenous and endogenous factors play an important role in spore germination and penetration of *S. graminicola*. Similar conclusions were made by Lucas and Knights (1987).

The results of this study also showed that germination of sporangia and zoospores on the leaf surface and penetration of leaves by the germ tubes decrease with the increase in leaf age (Fig.1, 2, and 3). This provides the basis for the decreased susceptibility of pearl millet plants to downy mildew with increase in the age as reported by Singh and Gopinath (1985). Similar phenomenon has been reported in several other host pathogen systems (Leu and Chu, 1959; Dalmacio and Exconde, 1969-70; Dickinson and Crute, 1974; Jones, 1978; Dange and Williams, 1980; Dernoeden and Jackson, 1980; Safeeulla and Shetty, 1980; Yeh and Frederiksen, 1980; Cohen, 1981; Stuteville, 1981; Cohen *et al.*, 1987; Rathore and Siradhana, 1988; Tuzen *et al.*, 1989; Patil *et al.*, 1992; Olanya *et al.*, 1993). It appears that the older leaf surfaces contain some substances which are inhibitory to zoospore germination and germ tube penetration. It has been reported that moisture film on the leaf surface inevitably carries both organic and inorganic substances (Brown, 1922; 1936) released from the underlying leaf tissue which have been shown to influence spore germination (Martin *et al.*, 1957).

The decrease in the percentage of successful penetration with increase in leaf age (Fig. 3) was due to the failure of germ tubes to penetrate. This failure in penetration could be due to the mechanical toughness of the cuticle. This is well supported by the linear increase in the thickness of cuticle and amount of wax from the 1st leaf to 8th leaf of all the genotypes tested (Fig. 7, 8 and 9).

Survival and infectivity of sporangia are largely influenced by the environmental factors, particularly temperature and humidity. Therefore, the ability of sporangia to cause epidemics is directly dependent on these factors. The processes that occur during the period between sporangial production and infection include dissemination of sporangia, zoospore release (Sporangial germination), zoospore germination, germ tube growth, and penetration. *In vivo* studies carried out, for the first time, showed that the sporangial and zoospore germination on the leaf surface, and penetration of the leaf by the germ tubes were highest at 20°C and decreased with the decrease or increase in temperature, being lowest at 35°C followed by 15°C on the leaf surfaces of both the resistant and susceptible genotypes (Fig. 4, 5 and 6). The optimum temperature for sporangial and zoospore germination on the leaf surface was 23.3°C for both types of genotypes. The optimum temperature for penetration was 24.3°C and 24.1°C, respectively for HB 3 (susceptible) and IP 18293 (resistant) (Table 7). It has been demonstrated by Singh and Gopinath (1990) that high levels of downy mildew developed only between 20 and 30°C. The absence of disease at 15°C and lower levels of downy mildew at 35°C have been observed. The results clearly show that at the above given temperatures, both germination and penetration were lowest.

At temperature less than 15°C or more than 35°C the disease will not occur in severe form.

The resistant genotypes greatly differed for per cent colonization by the pathogen. Colonization of *S. graminicola* was not observed in the leaf tissues of the resistant genotypes IP 18292 and IP 18293 at all stages of growth. In another resistant genotype, IP 18294, none of the 1st leaf samples were colonized, but colonization was observed in 10-20 per cent leaf samples from the higher growth stages. In resistant genotype, IP 18296, colonization was observed in 10-20 per cent leaf samples at all stages of plant growth. However, in both these cases, the growth of the mycelium was extremely restricted and there was no haustorial formation. In susceptible genotypes HB 3 and 7042 S, all the leaf samples from all the growth stages showed colonization (Table 8). These results confirm that despite some penetration by the pathogen in IP 18294, and IP 18296, these genotypes have shown stable resistance for 3 to 4 years in the field at several locations (Singh *et al.*, 1993) and should be extensively used in transferring resistance into elite cultivars.

The absence of mycelium in the resistant genotypes IP 18292 and IP 18293 was in agreement with the observations made by Lebeda and Reinink (1991) in lettuce. The observations on restricted colonization in IP 18294 and IP 18296 are also in line with the restriction of hyphal growth of downy mildew fungi reported in resistant varieties of other crops such as sorghum (Yeh and Frederiksen 1980),

maize (Shabani, 1978), sunflower (Mouzeyar *et al.*, 1993), and tobacco (Stolle *et al.*, 1988).

Based on the results in the present study, it is suggested that the specific reaction of the resistant genotypes is initiated after the penetration when hyphae begin to colonize the leaf tissue. There is a possibility for the production of antifungal substances by the host which could account for the restricted development of *S. graminicola* in the leaf tissues of resistant genotypes.

Leaf anatomical characters of pearl millet genotypes resistant and susceptible to downy mildew were worked out. It was found that the leaf cuticle was significantly thicker in the resistant genotypes than the susceptible ones at different developmental stages/leaf age studied. However, there were exceptions where no significant differences were observed between susceptible and resistant genotypes (Table 9). It was found that there was linear increase in the thickness of cuticle from the 1st leaf to 8th leaf of all the genotypes tested irrespective of their resistance and/ or susceptibility to downy mildew (Fig. 7 and 8). The decrease in the penetration by zoospore germ tubes from 1st leaf to 8th leaf of all the genotypes tested (Fig. 3) could be due to the increased thickness of the cuticle. Similar findings have also been reported in peach against powdery mildew (Weinhold and English, 1964), and in beans against *Rhizoctonia solani* (Stockwell and Hanchey, 1983), indicating that the thickness of cuticle increases with increasing leaf maturity.

From the above results, it can be inferred that the leaf cuticle thickness alone does not impart resistance. It probably plays some role in imparting resistance in the later stages of plant growth. This may be the reason that older leaves of even susceptible genotypes show resistance to infection by *S. graminicola*.

The three resistant genotypes (IP 18293, IP 18294 and IP 18296) possessed more wax, in some cases significantly more than the susceptible ones (HB 3 & 7042 S) (Table 11). Kumar (1974) reported that the wax content was high in the wheat varieties resistant to *A. triticina* and low in the susceptible one. Similar observations were reported in cases of tikka disease (*Cercosporidium personatum*) of groundnut (Gupta *et al.*, 1985), and leaf blight(*A. sesami*) of sesame (Gupta *et al.*, 1987). The results also show that the amount of wax on the leaf surfaces significantly increased from 1st leaf to 8th leaf of all the genotypes tested, irrespective of their resistance and/ or susceptibility to the disease (Fig. 9). The increasing amount of wax may be another factor that has helped in decreasing the germination of sporangia and zoospores from 1st to 8th leaf in all the genotypes tested (Fig. 1 and 2). Dickinson (1960) had discussed the possibility of the wax surface forming as the first barrier by repelling the water film required by a pathogen. Surface waxiness may affect the deposition of inoculum and a thick, waxy cuticle may limit the exudation of nutrients and other substances required by the pathogen in the initial stages of infection (Martin, 1964).

Same could be the case with the decrease in penetration of the pathogen (Fig. 3) and has been demonstrated in other cases. Gupta *et al.* (1987) opined that the higher amounts of wax on the leaf surface probably resist the penetration and establishment of *Alternaria sesami*. Berry and Mckeen (1985) also noted that *Nicotiana exigua*, highly resistant to *Peronospora hyoscyami* f.sp *tabacina*, escaped infection due to run off of infection droplets from the waxy adaxial epidermal surface and the sporangia that remained on the leaves usually produced abnormally long, twisted germ tubes which failed to penetrate.

However, in case of resistant genotype IP 18292, the wax content was found to be significantly less than all other resistant and susceptible genotypes. In addition, significant differences were not seen between the 3rd leaves of 7042 S, IP 18293 and between 3rd, 4th, and 6th leaves of 7042 S, IP 18294 and IP 18296 (Table 11). From these observations, it can be said that the amount of wax on the leaf surface may not play a direct role in determining the resistance or susceptibility reaction of a given genotype, but may indirectly contribute to the host plant resistance in the later stages of plant growth.

In the present study, significant differences between the number of stomata in susceptible and resistant pearl millet genotypes were not observed (Table 13). Similar observations were reported in pearl millet earlier (Meherunnisa Begum, 1984). The results also showed that there is no direct relationship between the number of stomata and the levels of resistance or susceptibility of pearl millet to downy mildew. However, per cent penetration through stomata is too low (Table

4) to make a meaningful conclusion. Royle and Thomas (1971) also found no correlation between the number of stomata per unit area of hop leaf surface and resistance to *Peronospora humuli*. Similarly, Subramanyam *et al.* (1982) found no correlation between rust resistance and number of stomata in groundnut.

In general, it was observed that the total phenols were significantly more in resistant genotypes than the susceptible ones at 7 and 11 days after sowing (DAS). However, the total phenols did not vary significantly between resistant genotypes IP 18292, IP 18293, IP 18296 and the susceptible genotype HB 3, 5 DAS (Table 14).

Satija *et al.* (1983), and Parashar *et al.* (1987) also reported that pearl millet varieties resistant to *S. graminicola* contained more total phenols than the susceptible varieties. The correlation between higher levels of phenols and resistance to downy mildew pathogens has also been reported in sorghum (Shetty and Ahmad, 1980), onion (Taleiva and Furst, 1985), and grape vine (Marutyan *et al.*, 1979).

The total phenols were more in non-inoculated leaves of resistant genotypes than in susceptible ones. Also, total phenols were significantly more in resistant genotypes than in susceptible ones in inoculated leaves, and there was an increase in total phenol content after inoculation in both the genotypes (Table 15). Muthusamy (1979) observed that the total phenols accumulated more in pearl millet leaves with infection. Kumhar *et al.* (1990) also noted that the total phenol contents were higher in shoots and roots of pearl millet infected by *S. graminicola*.

Similar observations were reported in sorghum (Shetty and Ahmad, 1980; Prabhu *et al.*, 1984), and grape vine (Srinivasan and Jeyarajan, 1977).

The total phenols increased with the increase in the age of the seedlings in all the genotypes irrespective of their resistance and/or susceptibility to downy mildew (Table 14). Increase in the total phenols with the increase in age can be correlated with the increase in resistance to infection by the downy mildew pathogen in the later stages of plant growth and development. The results are in agreement with those of Thukral *et al.* (1986) in pearl millet and Shetty and Ahmad (1980) in sorghum and maize.

The results of present study indicate that total phenols play a role in disease resistance. Since the phenols in leaves have been suggested as one of the factors for disease resistance (Farkas and Kiraly, 1962) and their presence in higher levels in leaves of resistant genotypes seems to confirm these findings. In the present study, significant differences in colonization were observed between resistant and susceptible genotypes. In resistant genotypes, either there was no growth of the mycelium, or it was extremely restricted, while in susceptible genotypes, the leaf tissue was colonized completely (Table 8). Several phenolic compounds are known to inhibit the growth of fungi in general (Goodman *et al.*, 1967; Kuc, 1964). Therefore, it is inferred from the present study that the restricted colonization in resistant genotypes is possibly related to the higher levels of total phenols present in resistant genotypes.

Qualitative differences in the total phenols were observed between resistant and susceptible genotypes. In case of resistant genotypes (IP 18292 and IP 18293) a distinct band was identified (phenol unknown) close to the solvent front which was absent in susceptible genotypes (HB 3 and 7042 S) (Plate 12). However, the isolation and chemical characterization of the compounds need to be done and the relationship between specific phenolic compound and resistance is to be ascertained. Further study in this direction will throw light in understanding the role of particular phenolic compounds in resistance phenomenon. Assabgui *et al.* (1993) found a negative, significant correlation between the amount of ear rot incidence in the field and the amount of (E)-ferulic acid detected in maize kernels by high pressure liquid chromatography. Sharma *et al.* (1983) reported that both coumarin and hydroquinone at a concentration of 10^{-3}M completely inhibit *in vitro* growth of *Cochliobolus heterostrophus*. Paper chromatography of the phenols extracted from 22 day old healthy plants indicated that hydroquinone (Rf=0.76) was present in both the maize inbred lines resistant and susceptible to leaf blight though in very low concentrations. Coumarin (Rf=0.85), was not detected in any of the inbred lines. Hildebrand and Schroth (1964) have provided evidence that disease resistance in pears is due to arbutin, a hydroquinone glucoside.

From the investigations on total soluble sugars (TSS) in resistant and susceptible genotypes at different stages of plant growth (Table 16), it is evident that the TSS did not show consistent relationship with resistance and/or susceptibility to downy mildew. This is in agreement with the results reported for pearl millet downy mildew by Rajaram Reddy (1978) and Parashar *et al* (1987).

Conversely, Alagianagalingam *et al.* (1978b) and Muthusamy (1979) observed more TSS in the leaves of susceptible pearl millet cultivars than in corresponding leaves of resistant cultivars.

In general, the TSS increased with the increase in the age of the seedlings, in all the genotypes tested (Table 16). These results are corroborating with those reported by earlier workers (Alagianagalingam *et al.*, 1978b; Bhatia and Thakur, 1994). The TSS did not differ significantly between inoculated and non-inoculated leaves in resistant genotypes, while in susceptible genotypes, the TSS were more in inoculated leaves than in non-inoculated leaves (Table 17). Mogle and Mayee (1981) also reported no alterations in sugar contents in a resistant line. They also observed increased reducing sugars and decreased starch in a pearl millet line susceptible to downy mildew. Muthusamy (1979) also found that the inoculation caused increase in TSS in the susceptible and resistant varieties, however, in the resistant varieties, the TSS increased in early stages and decreased in later stages. Bhatia and Thakur (1994) reported that TSS increased due to downy mildew infection in leaves of pearl millet.

In conclusion, the studies on host-parasite interaction provide the basis for understanding the mechanism(s) of resistance in pearl millet genotypes to downy mildew. Significant differences in cuticle thickness and amount of wax, that could explain the difference in resistant and susceptible genotypes were not observed but they might be playing a role in imparting adult plant resistance in both susceptible and resistant genotypes. The results show that the total phenols play

an important role in disease resistance and a more concentrated and valiant effort by future researchers on the isolation and chemical characterization of unidentified phenolic compound can add on to the important results emerged out of the present study.

SUMMARY

CHAPTER VI

SUMMARY

Studies pertaining to germination of sporangia and zoospores, penetration, and colonization of *Sclerospora graminicola* in susceptible and resistant pearl millet genotypes were carried out. Anatomical and biochemical differences between resistant and susceptible genotypes were also studied. All the studies were conducted at the Asia center of the International Crops Research Institute for the Semi-Arid Tropics (IAC), Patancheru, Andhra Pradesh, 502 324, India. The results are summarized below.

Sporangia germinated indirectly by producing zoospores. Occasionally, direct germination of sporangia by forming a germ tube has also been observed. The tip of the zoospore germ tube enlarged and formed an appressorium. In some cases, the germ tubes penetrated the leaves without forming appressoria. The germ tubes penetrated the leaf directly through the epidermal cells. Rarely, penetration through stomata was observed. The pathogen colonized the leaf tissue inter-and intracellularly.

Germination of sporangia and zoospores did not vary significantly among the three different regions (tip, middle, and basal parts) of the second leaf of a susceptible (HB 3) and a resistant (IP 18293) genotype. Germination of sporangia

and zoospores, and penetration by the germ tubes was significantly higher on the leaves of susceptible genotypes than the resistant genotypes. Germination of sporangia and zoospores and penetration by the germ tubes decreased with the increase in leaf age of both susceptible and resistant genotypes.

The sporangial germination, on the leaf surface of HB 3 (susceptible) and IP 18293 (resistant) was highest at 20°C & 25°C, where as zoospore germination and germ tube penetration was maximum at 20°C. Sporangial and zoospore germination and germ tube penetration decreased with the further increase or decrease in temperature.

Colonization was not observed in the leaf tissues of resistant genotypes IP 18292 and IP 18293 at all stages of plant growth. In resistant genotypes IP 18294, and IP 18296, however, 10-20 per cent leaf samples showed colonization at all stages of plant growth tested, except at 1st leaf stage in case of IP 18294. However, in both these cases, the growth of the mycelium was extremely restricted and there was no haustorial formation. In susceptible genotypes HB 3 and 7042 S, all the leaf samples showed colonization and haustorial formation, at all stages of plant growth.

A linear increase in the cuticle thickness (from the 1st leaf to 8th leaf), was observed on both the surfaces of all the genotypes. Cuticle thickness was significantly more in resistant genotypes than the susceptible ones. However, in

some cases, differences between the susceptible and resistant genotypes were not observed.

In the three resistant genotypes (IP 18293, IP 18294, and IP 18296), significantly more wax was observed than the susceptible genotypes (HB 3 & 7042 S). However, IP 18292, the most resistant genotype, showed significantly less wax than all the other resistant and susceptible genotypes at all stages of plant growth. A linear increase in the wax content was observed from the 1st to 8th leaf of all the genotypes.

Significant differences in number of stomata was not observed between the susceptible (HB 3 and 7042 S) and resistant (IP 18294 and IP 18296) genotypes on both the surfaces. Similar observations were also made between resistant genotype IP 18292 and susceptible genotype HB 3 on the upper surface.

The total phenols increased with the increasing age of the seedlings, in all the genotypes, and were found to be significantly more in resistant genotypes than the susceptible ones at 7 and 11 days after sowing (DAS). Significant differences were not observed among the three resistant genotypes (IP 18292, IP 18293 and IP 18296) and a susceptible genotype HB 3, at 5 DAS. Total phenols were more in resistant genotypes than the susceptible ones, both in inoculated and non-inoculated leaves. There was an increase in total phenols in both susceptible and resistant genotypes after inoculation.

Preliminary investigations were carried out to identify the phenolic compounds using thin layer chromatography (TLC), in disease free leaves of susceptible (HB 3 and 7042 S) and resistant (IP 18292 and IP 18293) genotypes in 1st leaf stage. In case of resistant genotypes, IP 18292 and IP 18293, a distinct band was identified close to the solvent front which is absent in susceptible genotypes, HB 3 & 7042 S.

Total Soluble Sugars (TSS) increased with the increase in the age of the seedlings, in all the genotypes tested and significantly more in susceptible genotype 7042 S than the resistant genotypes (IP 18292, IP 18293, IP 18294 and IP 18296) at 5, 7, and 11 DAS. However, significant differences were not found between 7042 S and IP 18293 at 5 DAS. Similarly significant differences were not observed between susceptible genotype HB 3 and resistant genotypes, IP 18292 and IP 18293, at 5 and 7 DAS; and between HB 3 and IP 18294 at 5 DAS. The total soluble sugars did not differ significantly between inoculated and non-inoculated leaves in resistant genotypes, while in susceptible genotypes, the TSS were more in inoculated leaves.

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