# CHARCTERIZATION OF LUPIN ANTHRACNOSE CAUSED BY *COLLETOTRICHUM GLOEOSPORIOIDES*

By

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## DECLARATION

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Dawit Solomon Ghebremariam

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## GENERAL INTRODUCTION

*Colletotrichun gloeosporioides* (Penz.) Penz. & Sacc. causes anthracnose on a wide range of hosts including, legumes and fruits, that are of particular economic importance (Manners *et al.*, 2000). Anthracnose of lupin (*Lupinus* spp.) is a destructive disease although consensus on the precise identity of the pathogen has not been reached (Gondran, 1994; Koch, 1996; Reed *et al.*, 1996; Yang & Sweetingham, 1998; Lardner *et al.*, 1999; Johnston, 2000). Resistance to anthracnose in cultivars of *Lupinus angustifolius* has been identified (Cowling *et al.*, 1999). Unfortunately *L. albus,* which is desirable for its higher yield and protein content, is very susceptible to the disease (Gondran & Pacault, 1997; Cowling *et al.* 1999)

The epidemiology of the disease (Gondran & Pacault, 1997) as well as variability in pathogen virulence and host-plant resistance, have been widely studied (Cowling *et al.*, 1999). Knowledge of the race composition of pathogens and their geographic distribution may help not only in the development of effective breeding programs, but also in averting the potential risk of crop damage. Monitoring isolates for possible shifts in pathogenicity may also help in the design of control strategies for the disease. An understanding of the mechanisms of penetration and infection of the pathogen will also be very useful in the development of sustainable disease control strategies (Bailey *et al.*, 1992). This applies particularly to pre- and post-penetration, the host-pathogen interaction and biochemical and physiological aspects of pathogenesis.

This thesis represents a compilation of four independent manuscripts based on research conducted over a period of two years. Each chapter is a separate entity within the framework of the lupin-*Colletotrichum gloeosporioides* interaction and intended to address certain relevant problems. The first chapter is a review of literature on the infection process of *Colletotrichum* species. The South African lupin industry relies on

cultivars of *L. angustifolius*. Any shift in the pathogenicity of *C. gloeoesporioides* is likely to be devastating to cultivation of the crop in South Arica. It is in this context that the second chapter sets out to assay the virulence of seven field isolates of *C. gloeosporioides* collected from the Western Cape Province, South Africa against 16 lupin cultivars.

Chapter 3 deals with the production of a gibberellin-like substance present in culture filtrate of *C. gloeosporioides* isolates collected in South Africa. The possible effect this may have on symptom expression is discussed. The fourth chapter of this thesis entails a preliminary examination of the infection process of *C. gloeosporioides* in lupin. Comparisons between histological reactions to the pathogen of susceptible (Kmutant) and less susceptible (Wonga) cultivars using fluorescence-light microscopy and scanning electron microscopy (SEM) are made. It is hoped that my study will contribute to a better understanding and knowledge of the infection process of lupin anthracnose and its control.

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# CHAPTER 1:

# A REVIEW OF THE INFECTION AND PENETRATION PROCESS IN THE GENUS *COLLETOTRICHUM*

## 1.1 INTRODUCTION

*Colletotrichum* Corda (anamorph) is a large fungal genus and one of the most important genera of plant pathogenic fungi in the sub-tropical and tropical regions of the world (Bailey *et al.*, 1992; Sutton, 1992). Its teleomorph is the ascomycete genus, *Glomerella* von Schrenk and Spaulding. *Colletotrichum* spp. cause pre- and post-harvest diseases on a wide range of plant species (Jeffries & Dodd, 1990) and have a significant impact on agriculture worldwide through their capacity to cause economic crop losses. Symptoms that are attributed to *Colletotrichum* spp. are commonly known as anthracnose and typically include depressed black lesions, which are subcuticular or angular and from which erumpent pink spore masses develop (Sutton, 1992).

An understanding of the processes that determine successful pathogenesis is a prerequisite for conducting biochemical and molecular studies relevant to plant pathology. The information can serve as a useful basis for fundamental research in physiological, genetic and molecular aspects of plant pathology that can be exploited by developing novel strategies for disease control (Skipp *et al.*, 1995). Knowledge of the entire infection process can provide information on which to base forecasting models and aid in the development of appropriate agricultural practices. It can also elucidate certain facets of the pathogen's life cycle that could be exploited in optimizing disease control strategies (Bailey *et al.*, 1992).

The present review deals specifically with the morphogenesis of infection structures and the penetration process pertinent to *Colletotrichum* species and presents recent findings related to these topics (Muirhead, 1981; Bailey *et al.*, 1992; Skipp *et al.*, 1995). Although an attempt will be made to review biotic factors that affect spore germination and appressorium formation, environmental factors that affect these processes, is beyond the scope of this review.

## 1.2 THE INFECTION PROCESS

### 1.2.1 Spore survival and dissemination

Two major sources of inoculum for *Colletotrichum* are conidia produced in acervuli and ascospores produced in perithecia (Bailey *et al.*, 1992). Sclerotia are also formed by *Colletotrichum truncatum* (Schw.) Andrus &Moore in culture and on soybean plants (Khan & Sinclair, 1992) and by *Colletotrichum coccodes* (Waller.) Hughes on tomato (Hornby, 1968). Sclerotia resemble old acervuli and their production allows the fungus to over winter in soil and crop debris (Khan & Sinclair, 1992). Dissemination of spores from young acervuli occurs via water droplets, whilst wind can distribute dry spore masses arising from older acervuli and ascospores (Nicholson & Moraes, 1980). Conidia and ascospores are encased in a moist hydrophilic mucilaginous matrix that maintains conidium viability by acting as a powerful anti-desiccant to ensure spore survival (Nicholson & Moraes, 1980; Ramadoss *et al.*, 1985; Louis *et al.*, 1988; Van Dyke & Mims, 1991). A high molecular-weight glycoprotein, which protects spores from desiccation, is the primary component responsible for the anti-desiccant properties of the mucilaginous matrix of *Colletotrichum graminicola* (Ces.) Wilson (Bergstrom & Nicholson; 1981; Ramadoss *et al.*, 1985). The germination rate of washed, matrix free conidia is negligible, whilst unwashed conidia germinate at the same rate as freshly harvested conidia (Louis *et al.*, 1988). Removal of the mucilaginous, water-soluble spore matrix from spores prior to storage at any relative humidity for 24 hrs results in a significant reduction in the viability of *C. graminicola* spores (Nicholson & Moraes, 1980).

Spores of many *Colletotrichum* species germinate readily after dispersal, but very poorly, or not at all, at high concentrations. This phenomenon, known as self-inhibition or auto-inhibition, occurs commonly enough among fungi to be considered a

general rule. It is thought to be an ecological adaptation that ensures spatial and temporal distribution of the species (Gottleib, 1973). The spore matrix also contains sufficient levels of inhibitors to prevent germination (Loius *et al.*, 1988; McRae & Stevens, 1990; Mondal & Parbery, 1992). An endogenous self-inhibitor, responsible for reduced germination under high concentration, was reported from conidia of *Colletotrichum gloeosporiodes* (Penz.) Sacc. *f. sp. jussiaea*, (Meyer *et al.*, 1983; Lax *et al.*, 1985). Chemical investigation of the self-inhibitor revealed dihydro-5-hydroxy-5 [(8 pentyl-2-oxocanyl)-acetyl)-2(3H)-furanone, for which the trivial name 'gloeosporone' was proposed (Meyer *et al.*, 1983). However, Mondal & Parbery (1992) indicated that 'gloeosporone' is mostly present in the spore matrix. Recently Tsurushima *et al.* (1995) re-examined the same isolate according to the procedure reported by Meyer *et al.* (1983), and could not detect 'gloeosporone'. The authors instead detected three active principles and identified these self-inhibitors as, (*E*) and (*Z*)-3-ethylidene-1, 3-dihydroindol-2-one and (2*R*)-(3-indolyl) propionic acid as CG-SI (*C. gloeosporioides*-inhibitor) 1, 2 and 3. The inhibitor, CG-SI 1 and 2 inhibited initial germtube emergence from conidia (Tsurushima *et al.*, 1995). Another inhibitor, 'mycosporine-alanine' prevents germination of *C. graminicola* within acervuli and ensures that ungerminated, viable spores can be dispersed across the leaf surface so that secondary infection can occur (Leite & Nicholson, 1992).

The importance of the matrix as a source of nutrients, energy and enzymes for germinating spores has often been demonstrated (Bergstrom & Nicholson, 1981; McRae & Stevens, 1990; Mondal & Parbery, 1992). Tu (1983) reported that the rapid decrease in viability of spores of *C. lindemuthianum* (Sacc. & Magn.) Br. & Cav. under wet conditions might be attributed in part to the loss of the mucilaginous water-soluble matrix of conidia. Studies of the composition of the matrix revealed the presence of

glycoprotein with carbohydrates and amino acids (Ramadoss *et al.*, 1985; Nicholson *et al.*, 1986) having a high affinity for phenols. This may represent one mechanism through which fungi inactivate toxic phenolic compounds in their environment (Nicholson *et al.*, 1986). Proline-rich proteins in the matrix of *C. graminicola* protect conidia from host-derived toxic phenolic compounds, including ρ-coumaric acid and ferulic acid that accumulate in water (Nicholson *et al.*, 1986, 1989). Water leachates from lesions were inhibitory to *C. graminicola* spores only in the absence of mucilage (Nicholson *et al.*, 1986). O'Connell *et al.* (1992) reported N-acetyl glucosamine (GlcNAc) and α-linked mannose or glucose residues depending on the fungal species. The materials appeared to be secreted by conidia during germination, since conidia were washed twice by centrifugation and suspension in water prior to applying them to slides.

The conidial matrix of certain *Colletotrichum* species plays an important role in the infection process itself because it contains enzymes that are known to be involved in the penetration of plant surfaces. Pectin esterase and cellulase (McRae & Stevens, 1990) have been reported in the conidial matrix of *Colletotrichum orbiculare* (Berk & Mont.) Arx. Invertase, cellulase and pectinase were detected in the conidial matrix of *C. gloeosporioides* (Louis & Cooke, 1985), while the conidial matrix of *C. graminicola* contained invertase (Bergstrom & Nicolson, 1981), β-glucosidase (Ramadoss *et al.*, 1985), cellulase and a non-specific esterase (Nicholson & Moraes, 1980; Ramadoss *et al.*, 1985). It is possible that these enzymes may cleave the phenolic esters and glycosides freeing the less water-soluble aglycones and making them more available for binding to the extracellular proline-rich proteins of the mucilage (Nicholson *et al.*, 1986). The production of larger lesions by older unwashed spores compared to washed spores, suggests that the matrix does indeed play a role (Mondal & Parbery, 1992). The addition of conidial matrix to inoculum of *C. orbicular* significantly increased

anthracnose development in *Xanthium spinosum* (McRae & Stevens, 1990) while removal of the matrix from inoculum of *C. gloeosorioides* reduced disease severity, the effect being attributed to a reduction in germination (Louis & Cooke, 1985).

It has been suggested that any inhibitory effect the spore matrix might have will be more pronounced at high spore concentrations, and more beneficial to mass spore survival than that of individual spores (McRae & Stevens, 1990). In spite of confirmation that the spore matrix can influence spore behavior, there is little knowledge of the mechanism involved or its ecological significance (Mondal & Parbery, 1992). The physiological basis for the role of the spore matrix was said to be at cellular and not sub-cellular level (Louis & Cooke, 1985). Although nothing is known of the persistence of the spore matrix following spore dispersal, it has been suggested that it may be attenuated to a stage where its effects are negligible (Louis & Cooke, 1985). In the absence of rain or impacting water droplets, mucilaginous spore masses become dry and are then wind dispersed (Louis *et al.*, 1988).

### 1.2.2 Spore germination and appressorial formation

#### 1.2.2.1 Spore germination

Ungerminated conidia are non-septate and single celled (Skoropad, 1967; Manadhar *et al.*, 1985; Smith *et al.*, 1999; Latunde-Dada *et al.*, 1996; Latunde-Dada *et al.*, 1999) but swell (Politis & Wheeler, 1973) and their nuclei divide mitotically prior to germination (Skoropad, 1967; Parbery, 1981;Van Dyke & Mims, 1991). Most germinating conidia have one septum (Jeffries & Dodd, 1990; Latunde-Dada *et al.*, 1996) but in some rare cases up to three septa have been observed (Skoropad, 1967; Latunde-Dada *et al.*, 1999; Smith *et al.*, 1999). Generally, *Colletotrichum* species begin to germinate 2-6 h after imbibition (Milholland, 1982; Van Dyke & Mims, 1991;

O'Connell *et al.*, 1993; Byrne *et al.*, 1997; Wei *et al.*, 1997; Pring *et al.*, 1995; Smith *et al.*, 1999). However, in *C. lindemuthianum*, germination was reported 18 h after imbibition on *Phaseolus vulgaris* (O'Connell *et al.*, 1985) and 12 h on French bean (Mercer *et al.*, 1975).

The number of germtubes produced and their position of emergence differ from species to species. Smith *et al.* (1999) reported that germtubes developed randomly from points on the conidia in *Colletotrichum dematium* (Fr.) Grove. The emergence of a single, lateral germtube near the end of a conidium was reported in *Colletotrichum truncatum* (Van Dyke & Mims, 1991), *C. gloeosporiodes* (Morin *et al.*, 1996), and *C. dematium* (Smith *et al.*, 1999). Skoropad (1967) reported that most spores of *C. graminicola* produce two short (1-2μm) germtubes from each end of the conidium although two or three germtubes from each cell have also been observed, especially when germination occurs on nutrient media.

### 1.2.2.2 Spore adhesion

Adhesion of an infecting spore is an essential component for fungal infection to occur and may be considered as a factor determining the virulence of many pathogens (Nicholson & Epstein, 1991). Adhesion ensures that a pathogen remains in contact with its host for as long as it is necessary for penetration to occur. It also firmly attaches the infection hypha to a site where penetration, whether mechanical or enzymatic, can be achieved (Bailey *et al.*, 1992).

The adhesion of *Colletotrichum* conidia occurs during a number of morphogenic stages. Conidia of *Colletotrichum* species can adhere to the host surface prior to germination (Young & Kauss, 1984; Mercure *et al.* 1994a) or after germination (Mercure *et al.*, 1994a, b; Sela-Buurlage *et al.*, 1991). Rapid adhesion increases the chances of disease development. In nature, conidia of *Colletotrichun* species are

dispersed in water droplets that easily run off leaf surfaces (Sela-Buurlage *et al.*, 1991). Louis *et al.* (1988) suggested that in the absence of rain or impacting water droplets, mucilaginous spore masses become dry and are then wind dispersed. Thus, early adhesion of ungerminated or germinated conidia ensures that propagules are not removed from the leaf (Mercure *et al.*, 1994b) or any other hydrophobic surfaces (Sela-Buurlage *et al.*, 1991) either by additional rainfall or by wind.

Considerable speculation has surrounded the role of the spore matrix in adhesion. In *C. graminicola*, removal of the mucilage does not prevent adhesion of ungerminated conidia to the host surface suggesting that it is superfluous (Mercure *et al,* 1994a, b). Evidence suggests that the adhesive material is released on contacting the surface of the host. *Colletotrichum* species display better adhesion on hydrophobic than hydrophilic surfaces (Young & Kauss, 1984; Sela-Buurlage *et al.,* 1991; Mercure *et al.*, 1994b). In *C. graminicola* (Mercure *et al.*, 1995) and *C. gloeosporioides* (Jones *et al.*, 1995), the release of protein exudates appears to consolidate the initial hydrophobic attachment of conidia. Ungerminated conidia of *Colletotrichum musae* (Berk. & Curt.) Arx. (Sela-Buurlage *et al.,* 1991; Mercure *et al.,* 1994a) and *C. graminicola* (Sugui *et al.,* 1998) apparently produced a proteinaceous adhesin. Proteinaceous adhesin from *C. musae* was produced more than once prior to germtube emergence, since conidia that were protease-E-treated and then washed, regained adhesiveness. Conidia that were maintained in suspension by constant mixing remained capable of adhesion to the substratum for several hours prior to germtube emergence. This may be due to new adhesive material being produced during this period (Sela-Buurlage *et al.,* 1991). Mercure *et al.* (1994a) reported that when conidia and germtubes were lifted from the host surface, a portion of the leaf cuticle in contact with the fungus was also removed.

Opportunities for detecting a broad range of molecules in the infection structures of *Colletotrichum* species have been provided by the use of lectin labeling and monoclonal antibodies (MAbs). By using MAbs, glycoproteins with some adhesive property have been found in extracellular matrix (ECM) produced by *C. lindemuthianum*. A set of glycoproteins that are very abundant in the fibrillar sheath surrounding germtubes were recognized by MAb UB22 (Pain *et al.*, 1992; O'Connell *et al.*, 1996) while UB26 recognized two high molecular weight glycoprotiens present on the surface of germtube and appressoria (O'Connell *et al.*, 1996: Pain *et al.*, 1996). MAb UB31 was also identified on ECM components specific to germtubes and appressoria (O'Connell *et al.*, 1996). The glycoprotein recognized by UB26 appears to be firmly attached to the fungal wall (O'Connell *et al.*, 1996). It adhered to the glass substratum on which the germtube grew to the extent that it could not be dislodged by ultrasonication (Pain *et al.*, 1996). UB27 also recognized 48-kDa glycoprotein present in the upper domed regions of plasma membrane of appressoria in *C. lindemuthianum* and was designated as *CLA1* (*C. lindemuthianum* Appressorium 1) (O'Connell *et al.*, 2000).

In *C. lindemuthianum* MAb, UB20, recognized a glycoprotein on the outer surface of the spore-coat and to a lesser extent at the plasma/cell wall interface (Hughes *et al.*, 1999; O'Connell *et al.*, 1996). Western blotting with UB20 showed 110 kDa as a major component of the glycoprotein. At a low concentration, UB20 inhibited attachment in an antigen-specific manner. Polystyrene microspheres bound selectively to the 110kDa glycoprotein in western blots, providing further evidence that this component could mediate interactions with hydrophilic substrata (Hughes *et al.*, 1999). O'Connell *et al.* (2000) reported that a plasmolysed appressorial cell remained in close contact with the host cell wall in the upper, domed region of cell showing that *CLA1*

glycoprotein may act as an integral molecule, binding the plasma memebrane to the cell wall. However, it remains unclear whether the retracted portions of membrane correspond precisely with the domain lacking *CLA1*.

Characterizing changes on the surface of *Colletotrichum* species during differentiation of infection structures *in vitro* and infection of host tissue using lectin cytochemistry is widely used (O'Connell *et al.*, 1992). Recently, lectin analysis showed a high molecular weight glycoprotein attached to a hydrophobic substratum using ECM released from conidia and germlings of *C. graminicola* (Sugui *et al.*, 1998). Earlier Marks *et al.* (1965) reported that a hyaline mucilaginous substance apparently held the lower appressorial wall firmly to the host cuticle in *C. gloeosporiodes*. Mature appressoria, for example, were not easily dislodged from the leaf surface of *Populus tremuloides* when washed with a gentle stream of water. Sugui *et al.* (1998) reported that the removal of appressoria of *C. graminicola* revealed an unstained gold/silver region surrounded by a zone that stained intensely for protein. The area that did not stain positively for protein appears to be the contact interface of the appressorium with the substratum. Examination at high magnifications revealed that the unstained area represents the site of the penetration pore. The association of carbohydrates with the secreted adhesin was demonstrated with different lectins. Mercure *et al.*, (1995) reported that the material released by ungerminated conidia of *C. graminicola* at the conidium-substratum contact interface contains glycoproteins. However, the clear zone that immediately surrounds conidia is composed of carbohydrates rather than protein as it was labelled by fluorescein isothiocyanate-concanavalin aggulutinin (FITC-ConA) and fluorescein isothiocyanate-*Lenis culinaris* aggulutinin (FITC-LCA) but not by gold/silver stain. Furthermore, since both these lectins bind to glucose and/ or to mannose, the results confirm that the material contains these two sugars.

Adhesive competence depends both on conidia and the leaf surface. There are few reports of the influence of conidium and leaf age on adhesion. Mercure *et al.* (1994a) reported a significant difference in the ability of 14-day-old conidia to adhere to the leaves of 5- and 8-week-old plants. Adhesion approached 30% of the conidial population by 30 min on 5-week-old plants. In contrast, adhesion of conidia on 8-week-old plants reached a maximum of only 20% by 2 h. The adhesive competence of ungerminated conidia decreased as conidia aged (Leite & Nicholson, 1992; Mercure *et al.*, 1994b).

Reports on the role of spore metabolism in adhesion in different species of *Colletotrichum* are conflicting. Sela-Buurlage *et al.* (1991) claimed adhesion is an active metabolic process, as conidia killed with UV light, formaldehyde or heat were significantly less adhesive than non-treated controls. Spore metabolism may also be required for conidia to remain adherent since adhered conidia became detached after exposure to a lethal dose of UV light (Sela-Buurlage *et al.*, 1991). Young & Kauss (1984) reported that respiratory inhibitors significantly reduced adhesion of *C. lindemuthianum* spores while Mercure *et al.* (1994b) reported that respiratory inhibitors had no apparent effect on the adhesion of *C. graminicola* conidia. Exposure of conidia to either low ($4^0$C) or high ($50^0$C) temperatures also did not significantly affect adhesion after 30 minutes. These findings suggest that active respiration may not be required for adhesion of ungerminated conidia.

Whether the adhesive material is released before or after conidia contact the substratum is open to question. In *C. graminicola,* Mercure *et al.* (1994a) proposed that adhesive was present pre- and post-adhesion. Autoclaving conidia prevented further production of adhesive but did not destroy the adhesive already present on the surface.

Furthermore, treatment of conidia with either a protein synthesis inhibitor or a glycoprotein synthesis and transport inhibitor significantly reduced adhesion.

### 1.2.2.3 Appressorial formation

All species of *Colletotrichum* produce simple appressoria although certain germinating spores do not produce appressoria (Porto *et al.*, 1988). Appressorium initiation may occur soon after germtube emergence, with some species producing a sessile appressorium (Morin *et al.*, 1996; Smith *et al.*, 1999). Germtubes sometimes also branch profusely to produce multiple appressoria (Latunde-Dada *et al.*, 1999) while subtended appressoria commence as a terminal swelling of the germtube and soon separate from the spore by means of a septum (Skoropad, 1967; Politis & Wheeler, 1973; Mercer *et al.*, 1975; Morin *et al.*, 1996).

Walls of appressoria are composed of an inner electron opaque layer and an outer electron lucent layer (Xuei *et al.*, 1988) and are usually a two- (Mercer *et al.*, 1975; Bell & Wheeler, 1986; Coates *et al.*, 1993) or three-layered structure (Mould *et al*, 1991a). Appressoria initially are hyaline but become darker when melanisation occurs (Muirhead & Deverall, 1981; Coates *et al.*, 1993; Morin *et al.*, 1996; Byrne *et al.*, 1997). Melanin provides a typical dark appearance and may protect them from irradiation, but the pigment may also play a crucial role in the penetration process (Katoh *et al.*, 1988; Howard & Ferrari, 1989). Melanin strengthens the appressorial wall to support the high internal hydrostatic pressure necessary for penetration of the plant cuticle and also determines the direction in which the infection peg emerges (Kozar & Neolitzky, 1978; Kubo & Furusawa, 1985). Dormant appressoria can withstand desiccation and high temperatures and ensures active growth when conditions are more favorable. The appressoria of *C. gloeosporioides* can survive at least 6 months on glass cover slips; but

when plated on potato-dextrose agar (PDA), they resume normal growth and eventually sporulate (Holmstrom-Rüddick & Mortensen, 1995).

Most species of *Colletotrichum* appear to form appressoria only when germtubes touch rigid surfaces. In *C. truncatum* germtube length is variable and appears to be related directly to moisture conditions, with wetter conditions favoring longer germtubes and on nutrient agar germtubes usually attain a considerable length before appressoria are formed, whereas on leaves, appressoria are formed immediately or after very limited elongation (Skoropad, 1967; Van Dyke & Mims, 1991). Dickman (2000) has reported *Colletotrichum trifolii* Bain & Essay. *ras* protein (CT-Ras) that regulates a signal transduction pathway that senses and responds to nutrients. Under nutrient-limiting situations a wild-type cells arrest vegetative growth (hyphal elongation) and differentiate (conidate), whereas cells expressing constitutively active CT-Ras continue vegetative growth, do not differentiate and, importantly, are impaired in polarized, systemic growth.

In *C. graminicola* (Politis & Wheeler, 1973) and in *C. trifolii* (Mould *et al.*, 1991a), each appressorium appears to contain a single nucleus with a prominent nucleolus packed with large number of free ribosomes, lipid bodies, mitochondria and glycogen before the appearance of the penetration peg. Mercer *et al.* (1975) reported only ribosomes and small lipid droplets in appressoria of *C. lindemuthianum.* In contrast, Skoropad (1967) reported the appearance of two nuclei in appressoria of *C. graminicola*, prior to their movement into penetration hyphae.

The lower portion of the appressorial wall contains a central germ pore, which provides an opening for the emerging infection peg/hyphae (Brown, 1977; Politis & Wheeler, 1973; Coates *et al.*, 1993). Dissolution of the appressorial wall to form the pore was reported in *C. graminicola* (Polities & Wheeler, 1973). The infection peg is

very thin when it passes through the cuticle, but it increases in diameter once inside the host cell (O'Connell *et al.*, 1985). It penetrates the host cell after emerging through the appressorial pore something, which has not been observed in all species (Politis & Wheeler, 1973).

An appressorial cone of varying shape normally surrounds the germpore and the wall of the infection peg appears to be an extension of the cone (O'Connell & Ride, 1990; Mould *et al.*, 1991a). It may act to focus hydrostatic pressure on the site of penetration (Wolkow *et al*, 1983) and could therefore, be considered as part of the infection hypha (Bailey *et al.*, 1992). Synthesis of the appressorial cone probably represents one of the initial stages in formation of infection hypha (Brown, 1977).

Ultrastructural studies have illustrated a fibrillar layer around the germtubes and mycelium of *Colletotrichum atramentarium* (Berk. & Br.) Taubenh (Griffiths & Campbell, 1973), *C. graminicola* (Kozar & Netolitzky, 1978) and *C. truncatum* (Van Dyke & Mims, 1991) as well as the germtubes and appressoria of *C. lindemuthianum* (O'Connell *et al.*, 1996). The walls of ungerminated and germinated conidia of *C. truncatum* (Van Dyke & Mims, 1991) are coated with fibrillar material. The spore coat in some cases appears to consist of preformed structures present in ungerminated, unimbibed conidia (Van Dyke & Mims, 1991). The maturation of appressoria was also associated with a thin, intensely fluorescent secretion of mucilage that was clearly distinct from, and external to, the melanized appressorium. The pattern of lectin binding to mucilage from different *Colletotrichum* species suggested the presence of polysaccharides or glycoproteins containing $\beta$-1, 4-linked GlcNAc, N-acetyl galactosamine (GalNAc) or galactose and $\alpha$-linked mannose or glucose residues (O'Connell *et al.*, 1992).

In *C. trifolii* on *Medicago sativa* L. (Mould *et al.*, 1991a; Mercer *et al.*, 1975) and in *C. lindemuthianum* on cowpea, *Vigna unguiculata* (Bailey *et al.*, 1990), appressorial maturation culminated in the migration of the conidial cytoplasm from the conidium to the appressorium via the germtube. When devoid of cytoplasm, the spore and germtube become vacuolated and conidia distal to the germtube appear as a non-staining 'ghost-cell' empty of identifiable cytoplasm, which subsequently collapses. Prior to penetration, vacuoles when present are small and scattered (Politis & Wheeler, 1973). In some *Colletotrichum* species, host penetration and colonization occurs immediately after appressorium formation. In other species, environmental conditions may be fungistatic prior (Binyamini & Schiffmann-Nadel, 1972; Parbery & Emmett, 1977) or post (Verhoeff, 1974; Chau & Alvarez, 1983; Coates *et al.*, 1993; Prusky *et al.*, 1998) penetration of the host. This aspect will be dealt with in para. 3.3.

### 1.2.2.4 Germination and appressorium formation

Emmett & Parbery (1975) suggested that in most species appressoria develop as a matter of course or as an end point to germination, provided the external environment at the plant surface is conducive to appressorium formation. Several factors interacting at the plant surface may inhibit or stimulate appressorium formation. A number of physical and chemical signals necessary for induction of appressoria formation have been reported. Various chemical substances associated with the plant surface are likely to exert an effect on the formation of appressoria. Chloroform extracts from sugar beet leaves on *C. acutatum* (Parbery & Blakeman, 1978) and anthranilic acid from banana on *C. musae* (Swinburne, 1976) stimulated appressorium formation. Anthranilic acid is converted to 2,3-dihydoxybenzoic acid that was usually the most effective stimulant. The possibility is that anthranilic acid is not a germination stimulant *per se* but active only because of its conversion to 2,3-dihydoxybenzoic acid (Harper & Swinburne, 1979)

Chemical stimuli present in epicuticular wax are implicated in conidial germination and appressorium formation. Conidia of *C. gloeoesporioides* are induced to germinate and differentiate to form appressoria by chemicals centered in the wax on the host surface (Prusky *et al.*, 1991; Podila *et al.*, 1993). Chemical signals are also emitted during fruit ripening by the hormone ethylene (Flaishman *et al.*, 1995). Solid contact surface is the principal requirement and stimulator for the morphogenesis of conidia from *C. gloeosporioides* (Kim *et al.*, 1998; Liu & Kolattukudy, 1998) and *C. trifolii* (Buhr & Dickman, 1997) in response to surface waxes and ethylene. Conidia resting on either a hard hydrophilic surface (glass) or a hard hydrophobic surface responded to the chemical signals only between 2 and 4 h after the initiation of contact with the hard surface (Flaishman *et al.*, 1995; Hwang & Kolattukudy, 1995). Using differential-display methods, *chip1* (*Colletotrichum* hard-surface induced protein 1 gene), which encodes an ubiquitin-conjugated enzyme, was expressed during the early stages (2 h) of hard surface treatment (Liu & Kolattukudy, 1998).

Cell signaling pathways operating in spore germination and appressorium formation of *Colletotrichum* species have been studied by monitoring the expression of genes encoding putative signaling components, or their inhibition. In *C. trifolii*, appressorium formation, in contrast to conidial germination and germtube formation, strongly depends on $Ca^{+2}$. The disturbance of calcium homeostasis, by ethylene-bis (oxyethylenenitrolo) tetra-acetic acid (EGTA) or calcium channel blockers, had a negligible effect on conidial germination and germtube growth, but markedly impaired appressorium development (Dickman *et al.*, 1995; Warwar & Dickman, 1996). Moreover, calmodulin (CaM) inhibitors affect both germination and differentiation implying that the $Ca^{+2}$/ calmodulin (CaM) signal transduction pathway is important in the early development of *C. trifolii* on the plant host surface (Dickman *et al.*, 1995;

Warwar & Dickman, 1996). Rather than a transient increase in $Ca^{+2}$, calcium cycling across the membrane is required for differentiation (Dickman *et al.*, 1995; Warwar & Dickman, 1996). RNA analysis of signal-transducing genes from *C. trifolii*, including genes for a serine-threonine kinase (TB3), calmodulin and protein kinase C, showed maximum transcription of all the three genes in conidia prior to or during germtube morphogenesis. TB3 and calmodulin gene transcription peaked during germtube morphogenesis (Buhr & Dickman, 1997). A putative ÇaM kinase (CaMK) cDNA of *C. gloeosporioides* was cloned with transcripts from hard surface treated conidia. The inhibition of this enzyme by KN93 (20 microM) inhibited germination and appressorium formation, blocked melanization and caused the formation of abnormal appressoria (Kim *et al.*, 1998). Yang & Dickman (1997) suggested the involvement of cAMP and cAMP-dependent protein kinase pathways. Both conidial germination and appressorial differentiation were impaired using specific inhibitors of cAMP-dependent protein. Using a pharmacological approach, including 8-Br-cAMP, sodium fluoride and 3-isobutyl-1-methylexanthine, all of which increase endogenous cAMP levels, showed the induction of appressorial differentiation on a non-inductive surface (1.5% water agar) (Yang & Dickman, 1997). In *C. trifolii* on alfalfa, a single copy gene (Ct-PKAC) encoding the catalytic cAMP-dependent protein kinase was isolated (Yang *et al.*, 1999). Transformants obtained through insertional activation of Ct-PKAC by gene replacement showed a small reduction in the growth relative to the wild type and conidiation patterns were altered. Importantly, PKA-deficient mutants could form appressoria, colonise wounded leaves and produce acervuli suggesting that loss of pathogenicity is most likely due to the failure in appressorial penetration (Dickman, 2000; Yang *et al.*, 1999). In *C. lagenarium CMK1* gene encoding a mitogen-activated protein (MAP) kinase regulates germination triggered by both glass surface contact and nutrient signals. However,

restoration of germination through the addition of yeast extract in *CMK1* mutant suggests that presence of a bypass pathway that can induce germination independent of *CMK1* pathways (Takano *et al.*, 2000). Although it was speculated that the synthesis or secretion of some extracellular materials shown to be involved in conidium attachment (Mercure *et al.*, 1995) might be impaired by disruption of *CMK1*, failure of germination in the *CMK1* mutant partially due to weak attachment of conidia cannot be excluded (Takano *et al.*, 2000). Kim *et al.* (2000) reported the cloning of a mitogen-activated protein kinase (MEK), designated as *CgMEK1*, from *C. gloeosporioides*. It was involved in a polarized cell division, with a preferential increase in F-actin in one of the daughter nuclei in response to hard surface contact, septum formation, germination and differentiation of germtube to appressoria. Disruption of this gene blocks hard surface-induced cytokinesis at a stage immediately following nuclear division. Thus, in the *CgMEK1*-disrupted mutants, the preferential increase in F-actin associated with one of the daughter nuclei and septum formation does not occur. This loss of polarity prevented germination and hyphal development and consequently loss of pathogenicity on its natural host. Instead, the *CgMEK1* mutants exhibited a budding-type of growth leading to the formation of oval cells. Although the exact sequence of signaling events needs to be further elucidated, the aforementioned evidence suggests that signaling pathways are involved during conidial germination and the differentiation of appressoria in *Colletotrichum* species.

Certain molecular events are triggered in *Colletotrichum* species by chemical signals from the host. Differential screening of mRNA from non-germinating appressoria that form conidia gave four cDNA clones representing transcripts found only in appressoria forming spores of *C. gloeosporioides* (Hwang *et al.*, 1995; Hwang & Kolattukudy, 1995). Two of these clones' *cap20* and *cap22* genes were uniquely

expressed during appressorium formation after 4 h exposure of spores to wax. Immunogold labeling with antibodies against *cap20* and *cap20* proteins showed that both of these gene products are located in appressoria (Hwang *et al.*, 1995; Hwang & Kolattukudy, 1995). Spores of *cap20* gene-disrupted mutants germinated and formed normal looking appressoria. Structural changes that might have resulted from the lack of *cap20* protein were not manifested in gross morphological alternations but showed a drastic decrease in virulence on avocado (Hwang *et al.*, 1995). It was concluded that *cap20* protein is necessary to make a functional penetration structure, the mechanism of which remains to be elucidated.

A number of external factors interacting on the plant surface are likely to influence appressorium formation. Species of *Colletotrichum* commonly fail to produce appressoria in the presence of abundant exogenous nutrients but germ tubes continue to grow and branch (Emmett & Parbery, 1975; Lenne & Parbery 1976). Mycelial development of *C. musae*, which is much greater on ripe bananas, presumably reflects higher concentration of nutrients in the leachates (Swinburne, 1976). The deprivation of both amino acids and sugars caused the formation of appressoria in *Colletotrichum acutatum* Simmonds (Blakeman & Parbery, 1977). A single factor operating alone, however, is unlikely to be the sole stimulus *in vivo*. Exposure to either of the two components caused the formation of long germ tubes without appressoria (Blakeman & Parbery, 1977). The requirement for exudates from ripe pepper fruits containing nutrients to stimulate appressorium formation in *Colletotrichum piperatum* Ell. & Ev. (Grover, 1971) may represent the exceptional adaptation amongst *Colletetrichum* species to enable infection of ripe fruits to take place. A high percentage germination and appressorium formation was observed on the leaf surface of *Stylosanthes guianesis*. Appressorium formation *per se* was neither inhibited nor enhanced and the number of

appressoria produced was directly related to the number of germinated spores (Lenne & Brown, 1991).

Microorganisms that inhabit the phylloplane may also stimulate the formation of appressoria. Species of *Bacillus* (Lenne & Parbery, 1976) and *Pseudomonas* (Blakeman & Brodie, 1977; Blakeman & Parbery, 1977), for example, have been shown to reduce germination but promote appressorial formation in *C. gloeosporioides* and *Colletotrichum dematium* (Pers. ex Fr.) *f. sp. spinacea*, respectively. Blakeman & Parbery (1977) suggested that *Pseudomonas* caused this effect by imposing nutrient stress on the fungus, since leaching nutrients from germinating spores could produce a similar effect. Lenne & Parbery (1976) reported a rapid lysis of the germtube and spores of *C. gloeosporioides* by the bacterium but an inability to lyase the appressorium.

The importance of protein synthesis for spore germination and appressorial formation by anthracnose fungi has also been investigated. Spores of *Colletotrichum lagenarium* (Pass.) Ell. & Halst. can germinate 40 min after the start of incubation even if protein synthesis is then inhibited by cycloheximide. Before this time however, protein synthesis is indispensable for spore germination (Furusawa *et al.*, 1977; Suzuki *et al.*, 1981). Morphogenesis of appressoria does not require *de novo* synthesis of protein after 40 minutes of incubation. When protein synthesis was completely inhibited by cycloheximide after one hour of incubation, appressoria matured in structure but not in function. Appressoria seemed to have no ability to penetrate artificial membranes (Suzuki *et al.*, 1981). In *C. trifolii,* conidial germination does not require *de novo* protein synthesis. Cycloheximide had no effect on spore germination but fungal growth ceased following germtube growth (Dickman *et al.*, 1995). Pascholati *et al.* (1993) showed that serine esterase inhibitors (Di-ispropyl flourophosphate-DIPF) have little effect on formation of appressoria by *C. graminicola* either on polystyrene or green leaf

tissue. Appressoria appear to mature even in the presence of DIPF but disease development does not occur. Suzuki *et al.* (1981, 1982a) concluded that while germling differentiation does not require protein synthesis once germination has started, appressorium maturation does. Cycloheximide, did not inhibit appressorium development, but inhibited the pigmentation of appressoria instead.

## 1.3 PENETRATION AND POST PENETRATION REACTIONS

### 1.3.1 Mode of penetration

*Colletotrichum* species penetrate the plant hosts through natural openings, through wounds and/or by direct penetration of the cuticle (Bonnen & Hammerschmidt, 1989a; Bailey *et al.*, 1992). The most common means of penetration is directly through the cuticle and epidermal cells. It usually occurs after formation of appressoria and the infection peg (Manadhar *et al.*, 1985; Latunde-Dada *et al.*, 1996; Morin *et al.*, 1996; Byrne *et al.*, 1997; Smith *et al.*, 1999). Direct penetration with an undifferentiated germtube has also been reported in *C. gloeosporioides* (Daquioag & Quimio, 1978; Manadhar *et al.*, 1985; Ogle *et al.*, 1990; Das & Bora, 1998).

Penetration through natural openings by *Colletotrichum* species has also been reported. Latunde-Dada *et al.* (1999) reported exclusive entry through stomata using isolate LARS 860 from cowpea preliminarily identified to be *Colletotrichum destructivum* O'Gara. This species used undifferentiated germtubes. While melanized appressoria were produced abundantly on the host surface, no infection hyphae were observed below them. *C. dematium* on onion (Russo & Pappelis, 1981), *C. acutatum* on guava fruit (Das & Bora, 1998) and *C. capsici* on cotton (Roberts & Snow, 1984) have been reported to penetrate through stomata, although these species are also capable of penetrating the cuticle and cell wall. TeBeest *et al.* (1978) reported penetration by appressoria of *C. gloeosporioides f. sp. aeschynomene* through the base of the numerous trichomes on the stem on northern joint vetch.

### 1.3.2 Mechanisms of penetration

Bailey *et al.* (1992) in their review mentioned the dubiousness of the mechanisms of penetration of the cuticle and epidermal cell walls as did Skipp *et al.*

(1995). Nevertheless, three mechanisms have been proposed: mechanical and enzymatic, either alone or acting synergistically.

Mechanical force as a mechanism of penetration was demonstrated by the indentation of a host surface associated with appressoria (Xuei *et al.*, 1988) or the infection peg (Mercer *et al.*, 1975) of *C. lindemuthianum* on the host cell wall. Mercer *et al.* (1975) reported that apart from a considerable increase in size of the infection hyphae, there was no visual evidence of cellulose degradation and other components of epidermal cell walls after penetration of the cuticle. Cutin degrading enzymes may thus not be involved (at least not as primary determinants) in penetration and mechanical force may be a more important factor involved in cuticular penetration by this fungus (Bonnen & Hammerschmidt, 1989a,b). In spite of an apparent inhibition of appressorial formation, conidia of *C. lagenarium* treated with inhibitors could still cause disease in etiolated cucumber hypocotyls. The addition of paraoxon, an inhibitor of cutinase, failed to affect the radial growth of *C. lagenarium* on PDA.

Mechanical penetration is supported by evidence from the research of several groups investigating the importance of melanization of appressoria in mechanical penetration. Research with *C. lindemuthianum* (Wolkow *et al.*, 1983), *C. lagenarium* (Kubo *et al.*, 1982; Katoh *et al.*, 1988) and *C. graminocola* (Pascholati *et al.*, 1993) suggests that melanized and structurally sound appressoria may provide the rigidity necessary to support the mechanical force required for penetrating the plant cuticle. Non-melanized appressoria of *C. lagenarium*, whether due to specific chemical inhibitors (tricyclazole) or mutation, resulted in a 90-95% reduction in the penetration of nitrocellulose membranes by the fungus (Kubo *et al.*, 1982; Suzuki *et al.*, 1982b; Katoh *et al.*, 1988).

Melanization of appressoria provides cell wall rigidity (Kubo *et al.*, 1982; Katol *et al.*, 1988; Kubo & Furusawa, 1991; Pascholati *et al.*, 1993) and also plays a role in osmosis by allowing a build up of internal hydrostatic pressure (Kubo & Furusawa, 1986; Howard & Ferrari, 1989) necessary for mechanical penetration. Colourless appressoria of the albino mutant of *C. lagenarium* germinated laterally on host plants or nitrocellulose membranes and consequently could rarely penetrate them. But addition of scytalone, a natural intermediate of melanin biosynthesis, isolated from the color mutant restored both appressorial pigmentation and penetration (Kubo *et al.*, 1982, 1983). Studies to elucidate the melanin biosynthesis pathways using melanin deficient mutants and homologues of known genes have shown that three melanin biosynthesis genes, notably *PKS1*, *SCD1* and *THR1*, have been cloned and characterized for *C. lagenarium* (Takano *et al.*, 1995; Kubo *et al.*, 1996; Perpetua *et al.*, 1996). Polyketide synthase (encoded by *PKS1*) is involved in the first step (Kubo *et al.*, 1991; Takano *et al.*, 1995). Subsequent steps consist of dehydration and reduction reactions. The dehydration of scytalone to 1, 3, 8-trihydroxynaphthalene (1, 3, 8-THN) and vermelone to 1, 8-dihydroxynaphthalene are performed by scytalone dehydratase (encoded by *SCD1*) (Kubo *et al.*, 1996). Reduction of 1, 3, 8-THN to vermelone is performed by 1, 3, 8-THN reductase (encoded by *THR1*) (Perpetua *et al.*, 1996). Melanin is then yielded by the polymerization and oxidization of 1,8-dihydroxynaphthalene. Takano *et al.* (2000) have confirmed that transcription of *PKS1* and *SCD1* was tightly linked to conidial germination process as regulated by CMK1 gene encoding a mitogen-activated protein (MAP) kinase but not to subsequent appressorium formation. *THR1* disappeared when conidia could not germinate but accumulated the same as the former two when conidia germinated. This was consistent with a previous report that *de novo* transcripts of the above three melanin biosynthesis genes accumulated 1 to 2 h after the start of conidial

incubation at $24^0$C, in advance to formation of appressoria and melanization (Takano *et al.*, 1997).

The role of enzymes during penetration has been examined and two lines of evidence have been used to support the role of cutinase in penetration and infections: a). specific organophosphate inhibitors of cutinase to block infection and b). antibodies raised against cutinase. Application of the potent cutinase inhibitor, di-ispropyl fluorophosphate, along with spores of *C. gloeosporiodes* prevented infection of the host (Dickman *et al.*, 1982). Pring *et al.* (1995) reported that the initial penetration of the cuticle of cowpea by *Colletotrichum capsici* (Syd.) Butl. & Bisby appears to be at least partially enzyme mediated as the penetration pore is well defined with no cuticular debris associated with penetrating hyphae. Similar results were obtained when antibodies to cutinase were included in spore suspensions (Dickman *et al.*, 1982). Neither the antibodies (Dickman *et al.*, 1982; Dickman & Patil, 1986) nor di-ispropyl fluorophosphate suppressed lesion formation when papaya's cutin barrier was breached by a needle prick prior to inoculation. These findings confirm that *C. gloeosporiodes* can penetrate the cuticular layer of papaya by secreting cutinase (Dickman *et al.*, 1982). At least for certain direct penetrating fungi, enzymes are less important than mechanical force. It is possible that cutin-degrading enzymes play a minor role in reducing the force necessary for penetration by loosening the cutin. This is an activity that may not be essential or detectable (Bonnen & Hammerschmidt, 1989a).

### 1.3.3 Latency

Latency can be defined as a quiescent or dormant parasitic relationship that a pathogen has with its host and which after a certain period converts to a pathogenic relationship (Muirhead, 1981; Coates *et al.*, 1993). The resumption of pathogenic activity generally happens during major changes in the host's physiological state such as

senescence of leaves, ripening of fruits, or wounding. Senescing leaves have almost completed their role in supplying photosynthate and are therefore of little further economic value but fruits increase in value during ripening. It is perhaps for this reason that latency has been studied more in fruits than in leaves (Muirhead, 1981).

In *Colletotrichum* species, the role of appressoria and infection hyphae as latent structures has often been debated and two forms of latency have been reported. Appressoria remain dormant on the surface of the fruit, giving rise to penetration hyphae and infection hyphae only at ripening (Binyamini & Schiffmann-Nadel, 1972; Parbery & Emmett, 1977). Another form of latency is when appressoria germinate immediately, penetrate, and produce a few latent subcuticular hyphae, which may resume activity during ripening (Verhoeff, 1974; Chau & Alvarez, 1983; Coates *et al.*, 1993; Prusky *et al.*, 1998).

The transition from a dormant parasitic relationship to an active one usually takes place only when fruits ripen, a phenomenon that is difficult to explain. Quiescent infection appears to be a fungal response to adverse physiological conditions temporarily imposed by the host. The lack of available nutrients, the presence of preformed antifungal compounds and the lack of enzymatic potential to penetrate have been tested as possible causes of quiescent fungal infection on unripe fruits (Prusky *et al.*, 1998). Binyamini & Schiffmann-Nadel (1972) suggested that the lack of development of *C. gloeosporioides* on avocado fruits while still on the tree might be because the hard exterior of the fruit contains fungal growth inhibitors. Verhoeff (1974) and Prusky *et al.* (1982) reported a preformed anti-fungal compound, 1-acetoxy-2-hydroxy-4-oxo-heneicosa-12, 15-diene (diene), associated with the resistance of fruits to attack by post-harvest pathogens. In freshly harvested fruits, the concentration of antifungal diene drops to subfungitoxic concentrations and then increases after a lag period to the

concentrations present in pre-harvested fruits (Karni *et al.*, 1989; Prusky *et al.*, 1991). The process leading to the fast decrease of the antifungal diene was suggested to be similar to the process occurring in normal ripening fruits, where the breakdown of the anti-fungal diene is catalyzed by lipoxygenase whose activity is regulated by the flavan 3-ol epicatechin (Prusky *et al.*, 1985, 1988). In freshly harvested fruit, a fast increase in epicatechin was observed, accompanied by an increase in lipoxygenase activity and consequently a decrease in the concentration of the anti-fungal diene (Karni *et al.*, 1989). Infiltration of fruits with α-tocopherol, an inhibitor of lipoxygenase activity, inhibited the decrease of antifungal diene and also the development of anthracnose lesions (Prusky *et al.*, 1983). The induction of epicatechin enhanced the level of the antifunfal compounds by preventing its breakdown. Induced levels of epicatechin occur by activation of the phenylpropanoid pathway with enhanced activity of PAL, CHS, and flavanone 3 hydroxylase. *Glomerela cingulata* (Stonem.) Spauld. & Schrenk also decays the mesocarp of peeled unripe fruits despite fungitoxic concentrations of the antifungal diene in idioblast oil cells. These idioblasts are metabolically active and can incorporate labeled precursors and synthesise and export diene (Prusky *et al.*, 1998).

Reactive oxygen species (ROS) induced by fungal infection of unripe fruits may modulate resistance, resulting in the inhibition of fungal development and quiescence. Both artificial inoculation of avocado pericarp tissue with *C. gloeosporioides* (*G. cingulata*) and treatment of avocado cell cultures with the cell wall elicitor of *G. cingulata*, increased the production of ROS, like hydrogen peroxide ($H_2O_2$). Unripe, resistant fruits are physiologically able to produce twice levels of ROS in pericarp tissue compared to susceptible tissue. Exogenous application of $H_2O_2$ at a rate of 1 mM to pericarp tissue enhanced ROS, phenylalanine ammonia lyase (PAL) activity, and epicatechin levels (Beno-Moualem & Prusky, 2000).

Treatment of host tissue after harvest with chemicals may cause nutrients to be released or made more available at the fruit surface. Ethylene increased or reduced the incidence of disease depending upon the time and concentration of the ethylene treatment and artificial inoculation. Upon exposure of inoculated green colored tangerines to ethylene, the infection process and resistance mechanisms were apparently initiated simultaneously, but despite this the infection process develops more rapidly (Brown & Barmore, 1977). The infection of tomato fruit by *C. gloeosporioides* did not proceed until the onset of ripening in response to ethylene. Compared with fruit from wild-type plants, infection progressed more slowly in transgenic fruit in which ethylene biosynthesis and ripening had been inhibited by an ACC oxidase (ACO) antisense transgene (Cooper *et al.*, 1998). Furthermore, 1-aminocylopropane 1-carboxylic acid oxidase 1(ACO1) mRNA, an enzyme responsible for the conversion of S-adenosyl methionine to ethylene, accumulated to maximum levels during the early stages of infection in ripe and unripe fruit. Ethylene biosynthesis increased rapidly in response to infection of ripe wild type and 1-aminocylopropane 1-carboxylic acid oxidase (ACO) antisense fruit but was 25% times greater in the former. Furthermore, ripening fruit from the mutant *ripening inhibitor* (*rin*), which are normally very resistant to infection, became infected quickly when incubated in the presence of ethylene, whereas fruit incubated in the absence of ethylene remained healthy.

Ethylene could possibly alter the quantity or quality of cellulolytic or pectolytic enzymes produced by *C. gloeosporioides*, which subsequently could affect its ability to colonize host tissue (Brown, 1975; Brown & Barmore, 1977). Transgenic tomato fruit deficient in polygalacturonase developed lesions at the same rate as the wild fruit type did to *C. gloeosporioides* infection. This suggests that depolymerization of pectic substances has little bearing on the progress of post harvest disease (Cooper *et al.*, 1998)

### 1.3.4 Infection strategies

*Colletotricum* species exhibit two main infection strategies, according to which species are loosely categorized. Skipp *et al.*, (1995) and Bailey *et al.*, (1992) have described the infection strategies employed by *Colletotricum* species and the reader is specifically referred to a review by Bailey *et al.*, (1992) for the list of *Colletotrichum* species and their respective infection strategies. Recent molecular aspects of these infection strategies will be dealt here *vis-a-vis* the aspects discussed in the aforementioned reviews. The first groups of species are known as intracellular hemibiotrophs; they penetrate both the cuticle and the epidermal cell wall and then invade the tissues. Some of the species in this group do not have a recognized biotrophic phase (Mould *et al.*, 1991a, b). In most cases, this group displays a two-phase infection process whereby the sequence of transient biotrophic phase followed by slow senescence and eventual death of the infected cells is repeated (Bailey *et al.*, 1992; Skipp *et al.*, 1995; Morin *et al.*, 1996). The pathogenicity and possibly the host specificity of hemibiotrophic *Colletotrichum* species may be determined during the early, apparently biotrophic stage of infection (Bailey *et al.*, 1992; O'Connell, *et al.*, 1993). The visible symptoms caused by *Colletotrichum* species are primarily a result of the later stage of necrotrophic development (Wei *et al.*, 1997).

During the first phase, the pathogen grows biotrophically and no symptom is produced on the plant. Immediately after penetration, infection vesicles grow and fungal hyphae then grow between the plasma membrane and plant cell wall. These vesicles give rise to primary hyphae, which also branch and grow rapidly until the initially infected cells are packed with convoluted mycelium (O'Connell *et al.*, 1985; O'Connell *et al.*, 1993; Skipp *et al.*, 1995; Latunde-Dada *et al.*, 1997; Smith *et al.*, 1999) or spread in to several nearby epidermal host cells (O'Connell *et al.*, 1985; Latunde-

Dada *et al.*, 1996; Wei *et al.*, 1997). Epidermal cells remain viable as shown by their ability to plasmolyse or accumulate neutral red stain (Wei *et al.*, 1997). Infected cells still plasmolyse normally (Latunde-Dade *et al.*, 1996; Wei *et al.*, 1997) and the host plasmalemma and tonoplast remain functional (Latunde-Dada *et al.*, 1996). This progressive biotrophic infection, which occurs in the absence of any visible symptoms, continues for several days prior to the production of lesion forming necrotrophic hyphae (O'Connell *et al.*, 1985). It has been suggested that the switch from biotrophy to necrotrophy is associated with a change in the metabolism and/or activity of cell wall-degrading enzymes (Wijesundera *et al.*, 1989).

The second phase is a visible destructive phase during which the pathogen grows necrotrophically. Secondary necrotrophic hyphae that are produced rapidly kill and destroy the tissue. The symptomless phase lasts for approximately two days (Bailey *et al.*, 1990; O'Connell *et al.*, 1993; Smith *et al.*, 1999) after which the second phase begins, leading to lesion formation, acervuli production and sporulation (O'Connell *et al.*, 1985)

Other *Colletotrichum* species are known as subcuticular intermural pathogens and penetrate the cuticle and grow beneath the cuticle causing a benign but often extensive subcuticular infection prior to the development of necrotrophic hyphae which grow through and destroy tissue (Bailey *et al.*, 1992). *Colletotrichum* species that have an initial subcuticular growth phase appear to attack a wide range of unrelated plant species (Bailey *et al.*, 1992; Sutton, 1992; Coates *et al.*, 1993; Pring *et al.*, 1995). *C. dematium* on onion, was shown by Russo & Pappelis (1981) to exhibit a sub-cuticular infection strategy with no detectable biotrophic stage observed.

### 1.3.4.1 Biotrophic phase

An intracellular biotrophic infection strategy may require a specific metabolic interaction with the host, thus limiting the host range. O'Connell (1987) reported that the interface in bean-*C. lindemuthianum* interaction lacks the structural and physiological specialization associated with infections by obligatory biotrophic fungi. Being a facultative parasite capable of growing on simple media, the fungus may scavenge sufficient nutrients from the plant apoplast for its initial development (O'Connell, 1987). The increased permeability of infected cells may also release more nutrients to the apoplast (O'Connell *et al.*, 1985). Certain glycoprotiens have been found to be present only when *Colletotrichum* is growing biotrophically and a set of fungal biotrophy genes is believed to promote pathogenic success during this stage (Green *et al.*, 1995).

Obviously, host range is an important consideration in the development of a mychoherbicide. Examining isolates for intercellular biotrophic infection strategy may be a useful screening tool for selecting *Colletotrichum* isolates with a narrow host range for a potential mycoherbicide (Wie *et al.*, 1997). Studies using MAbs in *C. lindemuthianum* isolated from bean show a protein epitope in a set of N-glycoproteins recognized by UB25 specific to wall of intercellular hyphae (IH) and interfacial matrix (Pain *et al.*, 1994; O'Connell *et al.*, 1996). These glycoproteins identified by UB25 were present in intercellular hyphae inside living host cells but not in primary hyphae growing in the intercellular space. These glycoproteins are only produced during the biotrophic phase of fungal development, when IH are in contact with living host protoplasts. They may be involved in functions specific to IH, such as plant fungal recognition or biotrophic nutrition (O'Connell *et al.*, 1996).

In Western blots, *UB25* recognizes a ladder of bands that are multiples of M(r) 40.5 kDa. A full length cDNA encoding the glycoprotein recognized by *UB25* has been isolated by expression cloning and designated *CIH1* (*Colletotrichum Intracellular Hypha 1*) (Perfect *et al.*, 1998). Northern blot studies of the infection process *in planta* have shown that *CIH1* is expressed by *C. lindemuthianum* in beans and by *C. destructivum* and *C. trifolii* in alfalfa during the biotrophic phase of fungul development. *CIH1* was also expressed by C. *lindemuthianum in vitro*, though not constitutively (Perfect *et al.*, 2000). *CIH1* is a fungal proline-rich glycoprotein that forms a multimetric structure in the interfacial matrix between the intercellular hyphae and the plant plasma membrane (O'Connell *et al.*, 2000). It resembles plant cell wall proline proteins (PRPs) and hydroxyproline-rich proteins (HRGPs) (Perfect *et al.*, 1998; O'Connell *et al.*, 2000). O'Connell *et al.* (2000) suggested that *CIH1* may function to prevent the plant recognizing the fungus, by mimicking plant PRPs and HRGPs and so presenting a 'pseudo' plant cell wall to the host. Furthermore *CIH1* glycoprotein could also act as a barrier to the host defense molecules or restrict the movement of fungal elicitors, enzymes or toxins.

Dufresne *et al.* (2000) identified a single open reading frame that encodes a putative transcriptional activator belonging to the fungal zinc cluster (Zn [II] 2Cys6) family designated as *C. lindemuthianum activator 1* (*CLTA1*). It is both a pathogenicity gene and regulatory gene involved in the switch between biotrophy and necrotrophy of the infection process of hemibiotrophic fungi. *CLTA1* disrupted strains failed in their pathogenicity but exhibited the same phenotype as the original ones. Complementation of these disrupted strains by *CLTA1* gene led to the full restoration of pathogenicity.

The expression of glutamine synthetase (GS), encoded by a single copy of DNA sequence pCgRL originating from a phylogenetically variable regions of the 25s rRNA

gene of *C. gloeoesporioides* on *S. guianesis*, increased markedly at an early stage of infection. At this stage, it was about six-times higher than during growth in rich culture media (Stephenson *et al.*, 1997). GS catalyses the condensation of ammonium ion and glutamate to yield glutamine in a reaction which requires ATP. This reaction is essential for the synthesis of glutamine and provides a mechanism for ammonia re-assimilation and detoxification (Stephenson *et al.*, 1997). Stephenson *et al.* (2000) reported that a nitrogen starvation induced *CgDN3* gene in axenic culture encodes a novel pathogenicity determinant associated with the biotrophic phase of *C. gloeosporioides* (*G. cingulata)* on *Stylosanthes guianensis*. The *CgDN3* gene most probably encodes a basic peptide of approximately 56 amino acids with no homology to any known protein and it was expressed at early stages of infection with transcripts detectable at 24 h after inoculation using RT-PCR technique (Manners *et al.*, 2000).

Mutants of *C. gloeosporioides* were produced by homologous recombination in which part of the coding sequence and promoter region of the *CgDN3* gene was replaced with a hygromycin-resistance gene cassette (Stephenson *et al.*, 2000) or disruption of the *CgDN3* gene (Manners *et al.*, 2000). The mutations lead to a loss of pathogenicity and elicitation of a hypersensitive response in the inoculated host. However, transformants mutated in the *CgDN3* gene have colonized a host tissue if placed on wounds (Manners *et al.*, 2000). This suggests that *CgDN3* gene product (secreted peptide) is either a molecular suppresser of a hypersensitive reaction during appressorial penetration of the host epidermis (Manners *et al.*, 2000; Stephenson *et al.*, 2000) or involved in a development program at primary infection that is essential to avoid triggering the host response (Manners *et al.*, 2000). Studies of the site of expression and the mode of action of *CgDN3* gene may provide clues as to how hemibiotrophic fungal pathogens suppress or avoid hypersensitivity during infection, hence crucial.

### 1.3.4.2 Necrotrophic phase

During penetration and colonization, *Colletotrichum* species secrete plant cell wall degrading enzymes that are thought to be primarily involved in the necrotrophic stage of pathogenesis. Sequential secretion of cell wall degrading enzymes has been demonstrated during the growth of fungal pathogens on isolated host cell walls (English *et al.*, 1970; Anderson, 1978). Pectin degrading enzymes that cause tissue maceration are the first enzymes produced by pathogens cultured on purified plant cell walls (English *et al.*, 1972; Anderson, 1978; Wijesundera *et al.*, 1989). Polygalacturonase (PG) and pectin lyase are thought to play an important role during pathogenesis by facilitating tissue colonization through the degradation of plant cell walls (English *et al.*, 1972). EndoPG (Wijesundera *et al.*, 1989) two forms of pectin lyase, α-and *β*-galactopyranosidase, α-arabinofuranosidase, and protease (Wijesundera *et al.*, 1984, 1989) are secreted by *C. lindemuthianum* in culture medium containing pectin or bean cell walls. Pectin lyase activity was first observed 4 days after artificial inoculation of bean with *C. lindemuthianum*, rising to a maximum after 7 days, after which activity declined (Wijesundera *et al.*, 1989).

Genes encoding pectin and pectate lyase have been cloned. A *pnlA* gene encoding pectin lyase from *G. cingulata* (Templeton *et al.*, 1994) and *pel* gene encoding pectate lyase from *C. gloeosporioides* on avocado (Wattad *et al.*, 1997; Yakoby *et al.*, 2000) has been isolated. Recently, to test the contribution of pectate lyase to promoting fungal pathogenecity, a pectate lyase gene (*pel*) from the avocado pathogen *C. gloeosporioides* (*G. cingulata*), isolate Cg-14, was expressed in *Colletotrichum magna* S. F. Jenkins & Winstead isolate L-2.5, a pathogen of cucurbits that cause minor symptoms in water melon seedling and avocado fruits. Transformed isolates showed additive maceration capabilities on avocado pericarp relative to the wild-type *C. magna*

alone, but did not reach the maceration ability of *G. cingulata*. However, more severe maceration and damping-off developed in watermelon seedlings inoculated with the transformed isolates compared with wild-type isolates, which showed no symptom development during the same period (Yakoby *et al.*, 2000). However, disruption of the *pnlA* gene did not affect pathogenesis of *G. cingulata* on pepper (*Capsicum* spp.) or apple (*Malus* spp.) indicating that the *pnlA* enzyme may not be essential for pathogenesis (Bowen *et al.*, 1995). Using gold-labelled polygalacturonase inhibiting protein (PGIP), endopolygalacturonase (endoPG) and associated cell wall fragment accumulation was visualized during infection for the first time (Benhamou *et al.*, 1991). EndoPG genes have also been reported in *C. lindemuthianum*. Southern analysis using *C. lindemuthianum* genomic DNA and the endoPG from *Cochliobolus carbonium* as a probe showed that at least two endoPG-encoding genes existed in the genome of *C. lindemuthianum* in axenic culture (Centis *et al.*, 1997). These genes were designated as *clpg1* and *clpg2* and have been cloned using degenerated PCR (Centis *et al.*, 1996, 1997). They show 61% similarity to each other (Centis *et al.*, 1996). Detection of endoPG transcripts by RT-PCR revealed that *clpg1*, but not *clpg2*, is expressed at the beginning of the necrotrophic stage of infection (Centis *et al.*, 1996, 1997; Dumas *et al.*, 2000). Strong expression of *clpg2* was detected 24 hr after inoculation, suggesting that this gene is expressed both *in planta* and *in vitro* rapidly before the onset of necrotrophic phase (Dumas *et al.*, 2000). The 5'-encoding region of *clpg2* was fused to the coding sequence of green fluorescent protein (GFP) to study the expression of the gene *in vito* in the presence of various carbon sources and pectin was the best inducer, and glucose had only a limited repression effect (Dumas *et al.*, 2000). During infection of the host, fluorescent germtubes and swelling appressoria were detected at the surface of the plant tissue, decreasing at the later stages and completely nil during the onset of necrosis

(Dumas *et al.*, 2000). Glucose, the main plant cell wall sugar residue present in the apoplast, prevented endoPG gene expression; partially when added to pectin at concentrations ranging from 5 to 10mM and, totally when added to arabinose at 55mM. It prevented the entrance of rhamnose and abolished the effect of arabinose on endoPG gene expression (Hugouvieux *et al.*, 1997). Northern blot experiments using specific probe for *clpg1* and *clpg2*, showed that only *clpg1* but not *clpg2* was induced by arabinose and rhamnose (Dumas *et al.*, 2000). The *Clpg1* gene is also involved in a direct elicitation of defense responses apart from encoding endoPG and its role in the necrotrophic stage of the pathogenesis (Dumas *et al.*, 2000).

Random insertional mutagenesis in *C. lindemuthianum* generated mutants that showed altered pathogenecity when tested on intact *Phaseolus vulgaris* seedlings, excised leaves and excised hypocotyls. Genomic and cDNA sequences suggest that the disrupted gene is a member of the A serine/threonine protein kinase family. A serine/threonine protein kinase-encoding gene, *clk*1 (*C. lindemuthianum* kinase 1) was weakly expressed in the mycelium of the wild-type strain grown on rich and minimal synthetic media but was undetectable during infection even when a sensitive reverse transcriptase-polymerase chain reaction methodology was used (Dufresne *et al.*, 1998).

In addition to the above enzymes, several low molecular weight, non-host specific phytotoxins isolated from cultural filtrates of *Colletotrichum* species have been discussed elsewhere (Bailey *et al.*, 1992). Recently Yoshida *et al.* (2000) reported the *in vitro* and *in planta* production of phytotoxins from *C. dematium*, a causal organism of mulberry anthracnose. The phytotoxin obtained from the diseased leaves induced necrosis in non-host plants leaves as well as in mulberry leaves. The toxins were also produced when the fungus was incubated in autoclaved mulberry leaves, suggesting that toxicity of the extract does not originate from plant response metabolites, such as

phytoalexins, but from fungal secondary metabolites. The chemical structure of the compound could not be identified owing to the low yield. Phytotoxins may be involved in pathogenesis, but their specific role and the mechanism in the host pathogen interaction remains to be elucidated.

## 1.4 RESISTANCE REACTIONS OF PLANTS TOWARDS *COLLETOTRICHUM* SPECIES.

Histological studies of *Colletotrichum* species on different hosts show various similarities in pathogen development. In both susceptible and resistant interactions, spore germination is followed by appressorial formation and penetration of the host epidermis. At this point there is either spread of infection hyphae in susceptible interactions or containment of fungal development in resistant interactions (Elliston *et al.*, 1976; O'Connell *et al.*, 1985; Churchill *et al.*, 1988; Baker *et al.*, 1989).

### 1.4.1 Hypersensitive reaction and phytoalexin accumulation

Race cultivar specific responses governed by genotypes of both the host and pathogen are mediated through the continued viability (compatible interaction) or death (incompatible interaction) of initially infected cells (Elliston *et al.*, 1976; Mercer *et al.*, 1974; O'Connell *et al.*, 1985). A true incompatible *Colletotrichum*-host interaction is characterized by rapid death and browning of the infected host cell known as the hypersensitive reaction (HP) (Bailey & Deverall, 1971; Elliston *et al.*, 1976). During compatible interactions, the infected cells do not die and the pathogen establishes a biotrophic relationship. However, when the necrotrophic phase is reached, the spread of secondary mycelium ceases or is restricted, resulting in a typical 'delimited' or 'restricted' lesion (Rahe, 1973; Mercer *et al.*, 1974; Rowell & Bailey, 1983; Latunde-Dada *et al.*, 1999).

Biochemical analysis has shown the accumulation of phytoalexin and phenolic compounds in lesions. Circumstantial evidence implicates the increased accumulation of phytoalexins (Bailey & Deverall, 1971; Anderson, 1977; Rahe, 1973; Bailey *et al.*, 1980; Latunde-Dada *et al.*, 1999) and phenolic compounds (Anderson, 1977; Stumm & Gessler, 1986; Xuei *et al.*, 1988; Baker *et al.*, 1989) in the restriction of fungal growth

during the HP reaction and lesion delimitation. In the *C. destructivum*-bean system, peak concentrations of phytoalexin coincided with a switch from biotrophic to necrotrophic development (Latunde-Dada *et al.*, 1999). The hypersensitive response of *P. vulgaris* cultivars to virulent races of *C. lindemuthianum* are characterized by a rapid activation of a number of defense genes, such as those encoding PAL, CHS and CHI in compatible interactions, a delayed increase of these mRNA coincides with the onset of lesion formation (Mahe *et al.*, 1993).

Cell wall elicitors from isolates of *C. gloeosporioides* (Prusky *et al.*, 1994) and bean-*C. lindemuthianum* compatible interactions (Mahe *et al.*, 1992) induced mRNA activity encoding the biosynthesis of PAL, CHI, Hydroxyproline-rich glycoproteins and CHS. Mahe *et al.* (1993) reported an earlier induction of PAL and CHT mRNA accumulation in incompatible interactions than in a compatible *C. lindemuthianum*-*Phaseolus vulgaris* pathosystem. Maximum accumulation, however, occurred almost simultaneously in both interactions. Extracellular components of *C. lindemuthianum* and citrus pectin fragments had synergistic effects on the accumulation of phenolics and phytoalexins in cotyledons of bean (Tepper & Anderson, 1990). Very limited additive or synergistic effects have, however, been observed between products of incompatible race and pectic fragments upon accumulation of mRNA for PAL and CHS (Tepper & Anderson, 1990).

Studies by Bailey & Deverall (1971) showed that phytoalexin accumulation occurs only in infected tissue and not in surrounding healthy cells. Rahe (1973) reported the accumulation of phaseollin predominantly at the edges of limited lesions. The concentration of phytoalexin in infected tissue is usually far higher than in tissue shown to inhibit fungal growth *in vitro* (Bailey & Deverall, 1971). Although germtube growth was also inhibited by small amounts of phaseollin, mycelial growth was less sensitive

and experiments indicate that phaseollin was metabolized by hyphae in liquid culture (Bailey & Deverall, 1971). The increased accumulation of phenolic compounds contributes to the fluorescent characteristic often associated with resistance interactions (Stumm & Gessler, 1986). These findings are consistent with the view that accumulated phytoalexins are responsible for restricting the growth of intercellular hyphae during resistant interactions. UV-absorbing and fluorescent compounds accumulate in resistant interactions of C. *trifolii* of alfalfa. Oh *et al.* (1999) reported PepCYP gene products play a role in defense mechanisms when the fungus invades and colonizes the epidermal cells of fruit in incompatible interactions. A PepCYP gene encodes a protein that is switched on when *C. gloeosporioides* invades and colonizes the epidermal cell layers of *Capsicum annuum*. The expression level of PepCYP in the epidermal cells is higher in incompatible interactions than in compatible interactions and then remains elevated in incompatible interactions. In compatible interactions, the expression is transient (Oh *et al.*, 1999).

### 1.4.2 Epicatechin and Polygalacturonase Inhibiting Protein (PGIP)

Prusky *et al.* (1989) reported that epicatechin inhibits endoPG produced by *C. gloeosporioides* and suggested that it might affect development of the fungus. At a concentration of 20 μg/ml, epicatechin (a flavan 3-ol present in unripe fruit-about 350 μg/g fresh weight) reduced the enzymes macerating ability of EPG by 64% (Wattad *et al.*, 1994). A non-pathogenic mutant strain of *C. magna*, which grows endophytically in watermelon, enhanced the resistance of avocado fruit (when sprayed as a challenge) to *C. gloeosporioides* by the induction of epicatechin (Prusky *et al.*, 1994).

PGIP present in host plants is involved in partial resistance to *Colletotrichum* by reducing the ability of endoPG to attack the cell wall, thereby preventing colonization of the plant tissue (Anderson & Albersheim, 1972; Lafitte *et al.*, 1984). The possibility that

PGIPs are involved in race-cultivar specificity is without evidence as inhibitors from distinct cultivar of *P. vulgaris* were equally able to inhibit endoPG from several races of *C. lindemuthianum* (Anderson & Albersheim, 1972; Lorenzo *et al.*, 1990). In incompatible interactions of *C. lindemuthianum* with *Phaseolus vulgaris* an early and rapid PGIP transcript accumulation was evident and this was associated with the HR. No accumulation occurred during the early stages of infection in susceptible lines, but a slight and delayed increase coincided with the onset of lesion formation (Nuss *et al.*, 1996).

### 1.4.3 Structural defence mechanisms

Various components present on the surface of invading pathogens can be recognized as elicitors activating the plant's defense mechanism. Recent studies indicate the existence of a rapid 'early' transient defense mechanism occurring mainly at the cell surface and without the activation of biosynthetic activity of cells (Wojtaszek *et al.*, 1995). Esquerre-Tugaye *et al.* (1992) in his review mentioned briefly the deposition of hydroxyproline-rich glycoproteins (HRGPs) from melon and alfalfa plants infected with *C. lagenarium* and *C. trifolii* respectively. The authors suggested modification of net charge of the host wall due to the polycationic nature of the HRGPs besides increase in the structural resistance. Elicitor molecules from *C. lindemuthianum* evoked the immobilization of salt extractable (hydroxy) proline-containing glycoprotein with O-linked oligosaccharide side chains (N-linked glycoproteins were not immobilized) from French bean possibly through the formation of a covalent cross link and subsequent structural barrier formation against pathogens. A good correlation appeared between the ability of given elicitor molecule to evoke immobilization of the salt extractable wall proteins and the level of production of oxidative burst ($H_2O_2$) and alkalinization of the culture media (Wojtaszek *et al.*, 1995).

A resumption of meristematic activity resembling a phellogen layer or heavy callus deposition were observed directly beneath appressoria when non-wounded attached banana fruit inoculated green with *C. gloeosporioides* and harvested at intervals up to full maturity (Stanghellini & Aragaki, 1966). Swelling of an undetermined chemical nature has been also reported directly beneath appressoria of *C. gloeosporioides* on epidermal cells of resistant mature leaves of *Populus tremuloides* Michx. (Marks *et al.*, 1965). The region of the host cell wall adjacent to the infection peg showed increased staining with lead citrate, and this was seen clearly in tissue fixed in the presence of tannic acid (O'Connell *et al.*, 1985).

In some host plants the production of papillae does not appear to be race specific and occurred with similar frequencies in both susceptible and resistant tissues in *C. trifoli-medicago sativa* (Mould *et al.*, 1991b) and *C. graminicola*-corn leaf (Polities & Wheeler, 1973) pathosystems. In the interaction of *C. graminicola* with oats (Politis, 1976), and *C. lagenarium* with immunized cucumber plants (Xuei *et al.*, 1988), the deposition of papillae under the penetration peg prevented pathogen ingress. In 5-15% of infections, hyphae were completely encased by papillae, and further growth did not occur (O'Connell *et al.*, 1985). The presence of numerous small vacuoles in the host cytoplasm adjacent to the papillae suggests the same components of the deposit may be conducted to the infection site by such vacuoles (Mould *et al.*, 1991b).

The synthesis of pathogenesis related (PR) proteins are also thought to be related to the defense of host plants to *Colletotrichum* infection (Lafitte *et al.*, 1993; Pinto & Ricardo, 1995; Regalado *et al.*, 2000). Daugrois *et al.* (1992) purified to homogeneity two $\beta$-1, 3-glucanases that are rapidly induced in the incompatible interaction between *Phaseolus vulgaris* and *C. lindemuthianum*. Pure endoPG from *C. lindemuthianum* race 3 elicited directly the biosynthesis of $\beta$-1, 3-glucanase in bean cuttings (Lafitte *et al.*,

1993; Dumas *et al.*, 2000) and that its action on host cell walls releases pectic fragments which can modulate hydroxy proline-rich glycoprotein gene expression. Pectin fragments released from susceptible bean cell walls only weakly elicited defense reactions compared to a resistant cultivar (Boudart *et al.*, 1995; Dumas *et al.*, 2000). The elicitation response was stopped by exoPG indicating that pectic fragments of critical size are required for endoPG-mediated defense in bean seedlings (Lafitte *et al.*, 1993). PR-10 protein was induced after infection of *Lupin albus* L. with *C. gloeosporioides* (Pinto & Richardo, 1995). Although *IF3* chitinase mRNA is a general response to stress in lupin, in young *Colletotrichum* infected necrotic *Lupinus albus* leaves the level of *IF3* chitinase mRNA was present in levels higher than that of PR-10 and rRNA (Lo *et al.*, 1999).

## 1.5 CONCLUSIONS

The present literature review has demonstrated the morphogenesis of infection structures and infection processes in *Colletotrichum* species. The conidial matrix has been shown to affect spore behavior although the mechanism involved is not clear. Spore adhesion is an essential component to fungal infection and is considered a virulence factor (Nicholson & Epstein, 1991). Conidial mucilage plays a crucial role in the survival (Nicholson *et al.*, 1986) and germination of spores (Nicholson, 1992). However, reports on the role of mucilage on adhesion (Mercure *et al.*, 1994a; Jones *et al.*, 1995) and the role of metabolism (Mercure *et al.*, 1994a; Sela-Buurlage *et al.*, 1991) in spore adhesion are not consistent. Additional research to elucidate these conflicting reports is thus required.

Differentiation of conidia of *Colletotrichum* into infection structures requires contact with a hard surface (Buhr & Dickman, 1997; Kim *et al.*, 1998; Liu & Kolattukudy, 1998) and specific host signals (Flaishman *et al.*, 1995; Hwang & Kolattukudy, 1995). The active involvement of signaling components operating in spore germination and appressorium formation of *Colletotrichum* species has been studied by monitoring the expression of genes encoding putative signal pathways (Dickman *et al.*, 1995: Warwar & Dickman, 1996; Buhr & Dickman, 1997; Yang & Dickman, 1997; Yang *et al.*, 1999). Although the involvement of signaling pathways on the differentiation of infection structures is well documented, it is only in a few cases that the exact sequence of signaling events and the underlying mechanisms of operation are explained (Kim *et al.*, 2000) or speculated (Takano *et al.*, 2000). Much research still needs to be done in this area to arrive at novel targets for strategies to protect plant from fungal infection.

In recent years, genes required for pathogenicity have been identified in *Colletotrichum* species using several molecular techniques. Chemical signals from the host surface trigger genes (*cap20* and *cap22*) during spore germination and appressoruim formation (Hwang *et al.*, 1995; Hwang & Kolattukudy, 1995) that enable the pathogen to produce functional appressoria. During the biotrophic phase of fungal development, genes encoding a fungal proline-rich glycoprotein (*CIH1-Colletotrichum Intracellular Hypha 1*) (Perfect *et al.*, 1998; O'Connell *et al.*, 2000; Perfect *et al.*, 2000) and putative transcriptional activator belonging to the fungul zinc cluster family (*C. lindamuthianum activator 1-CLTA1*) (Dufresne *et al.*, 2000) have also been cloned. Because the glycoprotein encoded by *CIH11* resembles plant cell wall proline proteins (PRPs) and hydroxyproline-rich proteins (HRGPs) (Perfect *et al.*, 1998; O'Connell *et al.*, 2000), it was suggested that *CIH1* may function to prevent the plant recognizing the fungus, by mimicking plant PRPs and HRGPs and so presenting a 'pseudo' plant cell wall to the host. Furthermore *CIH1* glycoprotein could also act as a barrier to the host defense molecules or restrict the movement of fungal elicitors, enzymes or toxins (O'Connell *et al.*, 2000). Gene disruption analysis revealed that these genes are essential for pathogenicity. However, further work still needs to be done in terms of obtaining similar genes and elucidating the mechanism whereby the gene produces functional infection structures.

Penetration and colonization of a plant host by *Colletotrichum* species is aided by cell wall degrading enzymes that are thought to be primarily involved in the necrotrophic stage of pathogenesis. Several low molecular weight, non-host specific phytotoxins have also been isolated from cultural filtrates of *Colletotrichum* species (Bailey *et al.*, 1992) and *in planta* (Yoshida *et al.*, 2000). Yoshida *et al.* (2000) reported that phytotoxins obtained from diseased leaves induced necrosis in non-host plants

leaves as well as in host plants. However, the literature does not mention their specific role or the mechanisms by which they became pathogenic. Further consolidation of the production of phytotoxins in live plant tissue and their specific role in host pathogen interaction is therefore still important.

Lupin anthracnose caused by *C. gloeosporioides* (Penz.) Penz. & Sacc. (Weimer, 1943; Sweetingham *et al.*, 1995; Reddy *et al.*, 1996) is a devastating disease worldwide (Baer *et al.*, 1999; Paulitz *et al.*, 1995; Gondran, 1994; Frencel, 1998; Ageav, 1993; Savchenko *et al.*, 1994; Sweetingham *et al.*, 1995; Koch, 1996; Dick, 1994). The main features of the disease are lupin plants with bent stems and dark brown, elongated lesions in the crook of the bend (Sweetingham *et al.*, 1995; Murray, 1997). Diseased stems collapse, displaying characteristic necrotic, crook shaped distortion (Frencel, 1998). The South African lupin industry relies on *L. angustifolius* cultivars. Any shift in pathogenicity of *C. gloeoesporioides* will therefore be devastating. The second chapter of this paper sets out to investigate shifts of virulence among field isolates of *C. gloeosporioides* from the Western Cape Province, South Africa.

Despite the seriousness of the pathogen, biochemical and physiological aspects involved in the infection process have not been investigated. A preliminary study was made to ascertain whether *C. gloeosporioides* produces a gibberellin-like substance *in vitro* that could possibly be used in the screening for disease resistance.

Understanding the mechanisms of penetration and infection of this pathogen would be helpful for the development of disease control strategies. However, histological studies of anthracnose disease of lupin have not been reported. The fourth chapter of this paper will shed some light on the infection process of *C. gloeosporioides* in lupin and compare the histology of compatible (Kmutant) and incompatible reactions (Wonga) using fluorescence light microscopy and scanning electron microscopy.

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# CHAPTER 2

## PATHOLOGICAL CHARACTERIZATION OF THE SOUTH AFRICAN POPULATION OF *COLLETOTRICHUM GLOEOSPORIOIDES* ASSOCIATED WITH LUPIN ANTHRACNOSE TO SIXTEEN LUPIN CULTIVARS

## ABSTRACT

Lupin anthracnose caused by the fungus *Colletotrichum gloeosporioides* is a worldwide problem in the cultivation of lupins (*Lupinus* spp.). The disease has been present in South Africa since 1993. To aid plant breeders in breeding programmes it is necessary to establish whether any pathological shifts within the South African population of the pathogens have occurred. Six strains of the fungus from different localities were compared with an isolate collected in 1995 when the disease first established itself in the Western Cape Province, South Africa. Fourteen-day-old seedlings of a set of 16 lupin cultivars with different genetic backgrounds were spray-inoculated in the glasshouse with the seven fungal isolates. Disease severity was rated 14 days after inoculation. No pathological differences were detected within the fungal population. The most susceptible selection was *L. albus* (cv Kiev Mutant) while *L. angustifolius* (cvr Wonga and Tanjil) were the most resistant. Although no pathological differences within the current fungal population were detected, regular screening to assist the local breeding programme should continue.

## 2.1 INTRODUCTION

Lupins (*Lupinus* L.) are susceptible to numerous plant pathogens and anthracnose is currently the most devastating fungal disease of lupins worldwide [Chile (Baer *et al.*, 1999); North America (Paultiz *et al.*, 1995); Europe (Gondran, 1994; Frencel, 1998); Russia (Ageav, 1993; Savchenko *et al.*, 1994); Australia (Sweetingham *et al.*, 1995); South Africa (Koch, 1996) and New Zealand (Dick, 1994)]. Bending or twisting of the stems often characterizes the disease and eventually the whole stem curls up and the entire plant becomes distorted. Elongated oval brown lesions of 0,5 to 2.0 cm or larger occur in the curves. The center of lesion is creamy to pinkish-orange due to the production of masses of slimy spores. The leaves and floral parts are also attacked and round lesions are formed on the pods, from which the fungus spreads to the seed. Distinct lesions are not formed on the seed, which make it difficult to detect fungal infection (Sweetingham, 1997).

Confusion regarding the identity of the causal species of *Colletotrichum* Corda. still exists. Some claim the disease is caused by *C. gloeosporioides* (Penz.) Penz. & Sacc (Gondran, 1994) or *C. acutatum* J. H. Simmonds ex J. H. Simmonds (Reed *et al.*, 1996), while others have produced evidence that it might be a separate species (Koch, 1996) or *formae specialis* of *C. gloeosporioides* (Yang & Sweetingham, 1998) or *C. acutatum* (Lardner *et al.*, 1999; Johnston, 2000). For the purpose of this study, the causal fungus will be referred as *C. gloeosporioides*.

An anthracnose disease of *L. angustifolius* L. in the United States was described in 1943 by Weimer (1943). Due to the absence of reference cultures it could not be established whether the same fungus causes the current disease. *L. angustifolius* (cv. Rancher) resistant to the 1943 disease tested susceptible to isolates of the current anthracnose causing *Colletotrichum* sp. (Cowling *et al.*, 1999)

Today lupin anthracnose affects not only the production of *L. angustifolius* L. (Sweetingham *et al.*, 1995) but also *L. albus* L. (Gondran, 1994), *L. arboreus* Sims (Dick, 1994), *L. luteus* L. (Sweetingham, 1997), *L. consentinii* Guss (S. H. Koch, Pretoria –personal communication), *L. mutabilis* (Baer *et al.*, 1999) and *L. polyphyllus* Lind. (Reed *et al.*, 1996). In South Africa, the disease was first recorded during the summer of 1993/1994 in the Free State Province (Koch, 1996). It has since spread to the Western Cape Province, the main lupin producing area in South Africa where lupin production has been reduced by two thirds since the outbreak of the disease in 1996 (Koch, 1999). In Australia, the cultivation of *L. angustifolious* L. is especially difficult in high rainfall areas owing to this disease (Cowling *et al.*, 1999). The successful cultivation of lupin in South Africa is very dependent on the natural resistance of certain cultivars to pests and pathogens.

Studies on the epidemiology of the disease (Gondran & Pacault, 1997) and one attempt to determine pathological variability in pathogen across the world were undertaken by Cowling and co-workers in different countries including South Africa (Cowling *et al.*, 1999). Despite differences in trial methodology and climatic/environmental conditions during the trials no major differences between the respective populations or isolates of the fungus were detected. The South African lupin industry is, for the first time during the last decade, making a comeback after powdery mildew devastated production in the early seventies. However, since the outbreak of the anthracnose in 1993 (Koch, 1996), unpredictable weather conditions and a decline in lupin production have made it difficult to determine possible shifts in pathogen variability under field conditions. Both the South African and Australian lupin industry rely on more resistant *L. angustifolius* cultivars to combat anthracnose. Any shift in pathogenicity of *C. gloeoesporioides* will therefore be devastating to both countries.

The present study was launched to determine shifts in virulence within the current population of *C. gloeosporioides* associated with lupins in South Africa compared to that which existed in 1995 following the first outbreak of disease in 1993.

## 2.2 MATERIALS AND METHODS

### 2.2.1 Collection of isolates and inoculum preparation

Diseased stem pieces or pods showing typical anthracnose lesions were collected from lupin fields where the disease occurred (Table 2.1). Isolates were collected from different areas where disease occurred between 1999 and 2000. Pieces of diseased plant material were surface disinfected for 2 min with 1 % NaOCl solution and rinsed three times with sterile distilled water. Small pieces of tissue from the disinfected surface (2 $mm^3$) were incubated on potato dextrose agar (PDA) supplemented with streptomycin (0,05 g/1000 ml) and chloramphenicol (0,05 g/1000 ml). All cultures identified as causing lupin anthracnose were single-spored and cultures were maintained on potato carrot agar (PCA) slants at 5 $^{o}$C.

Two sets of isolates SHK 2141, SHK 2148 SHK 2140 and SHK 2153, SHK 2154 and SHK 2155 (Table 2.1) were used to inoculate plants in two respective trials (1 and 2). Isolate SHK 1033 collected in 1995 in the Western Cape and was used in both trials as a reference. Fungal cultures were cultivated on PDA at 23 $^{0}$ C under a 12 h light/darkness regime. Conidia were dislodged in sterile distilled water and the conidial suspension was adjusted using a haemocytometer to 5 X $10^{5}$ conidia/ml. Two drops of Tween 20 were added to the conidial suspension.

### 2.2.2 Soil preparation and planting

Sixteen lupin accessions with varying genetic backgrounds (Table 2.2), of which some are currently being evaluated in the National Lupin Cultivar Trials, were selected. Lupin seeds were surface disinfected for 10 minutes in a 1% NaOCl solution and then rinsed three times with sterile distilled water. Eight surface disinfected seeds per cultivar were planted in a 128 well seedling tray (3 x 3 x 10 cm) containing a 1:1 mixture of virgin loam soil and perlite (Genolite). Seeds were covered by 5 mm of sand. A split plot design with isolates as the main plot and cultivars as subplot was used. Each treatment was replicated four times. The seedlings were cultivated for 14 days in a glasshouse at 20 $^{\circ}$ C $\pm$ 2 $^{\circ}$ C and plants were watered regularly. Prior to inoculation, plants were watered thoroughly for seven days to maintain moisture levels.

### 2.2.3 Inoculation

Seedlings were sprayed until run-off with the respective conidial suspensions and each tray was covered with transparent plastic in order to maintain humidity levels close to 100 %. Treatments were separated by means of plastic dividers to prevent cross-contamination. To further simulate conditions conducive to disease development, the roof of the glasshouse was covered with 80% shade cloth (Alnet $^{TM}$). Within the glasshouse, an additional layer of 50% shade cloth was placed 1 m above the inoculated seedlings to further protect them from the sun. After five days, holes approximately 4 $cm^2$ were made in the plastic covering to begin hardening–off of the seedlings. Two days later, the plastic covering was removed from all trays. The seedlings, walls and floor of the glasshouse were regularly sprayed with a fine mist of water to maintain high humidity levels for an additional five days.

### 2.2.4 Disease assessment

Fourteen days after inoculation, when symptoms were clearly discernible, each seedling was scored for disease severity on a scale ranging from 0 to 5 (0 = plants healthy, no symptoms and 5 = dead plants) (Fig 2.1). The mean for each replication per accession was calculated and means were combined. Disease severity indices for each accession per treatment were calculated and data were analyzed using the statistical program GenStat (GenStat, 2000). Treatment differences were tested by way of analysis of variance (ANOVA) and Fisher's test for least significant difference (LSD) was used to separate means at the 5 % level of confidence (Snedecor & Cochran, 1980). The most tolerant and susceptible groups of cultivars with a probability of 95% were selected according to the multiple t-distribution test procedure of Gupta & Panchapakesan (1979).

## 2.3 RESULTS

Disease severity ratings ranged between 1.7 to 4.0 and 1.6 to 4.0 in trial one and two respectively (Table 2.2). The most susceptible and most resistant genotypes were grouped according to Gupta and Panchapakesan's multiple t-distribution test procedure (Gupta & Panchapakesan, 1979) (Table 2.2). Accessions differed significantly in anthracnose severity ($P < 0.001$) in both trials. All varieties displayed consistent levels of tolerance/susceptibility against all isolates tested in both trials. *L. angustifolius* cv's Wonga and Tangil were superior to all other cultivars with the lowest severity score. In both trials, *L. albus* cvs. CED 6150, Esta, Swartland, Kiev Mutant, Vladmir and Hamburg appeared to be consistently susceptible to all the isolates. A moderate level of resistance was observed in *L. luteus* cv Wodjil, Markiz, Juno and *L. angustifolius* cv's Môredou and Merrit. Not one of the accessions were totally resistant to the disease.

There was no significant interaction between isolates and cultivars (P = 0.307 and 0.206) in both trials (Table 2.2, 2.3 & 2.4). In addition, no significant differences between the seven isolates used in this study were displayed.

## 2.4 DISCUSSION

Breeding programmes are long-term investments aimed at obtaining the most resistant cultivar of a plant species within the shortest period of time. Breeders require fast reliable methods to tests lines for resistance and must therefore use the most pathogenic strains of the pathogen responsible. As soon as more pathogenic strains are detected in the field, breeding programmes must be adapted to include these strains. Yang & Sweetingham (1998) established that there was little variation based on vegetative compatibility between the isolates of *Colletotrichum* from lupin throughout the world. Most of the isolates, including the South African isolates, belonged to the vegetative compatibility group 2 (VCG2). Vegetative compatibility is not strongly linked to pathological differences within a specific host species, but to host species attacked (Ploetz & Correl, 1988; Strausbaugh, 1993; Appel & Gordon, 1994; Katan *et al.*, 1994; Mess *et al.*, 1994). Physiological races of the pathogen exist within VCG's. Within the genus *Colletotrichum*, many examples of physiological races within species exist, for example *C. lindemuthianum* on beans (*Phaseolus vulgaris* L.) (Barrus, 1911; Koch, 1996). It is therefore important to constantly be on the lookout for the appearance of races that might overcome resistance in commercial cultivars.

Recently collected isolates of the fungus were compared to ones collected when the disease first established itself in the Western Cape Province. In our results, based on the disease severity expressed by the seven isolates on 16 different lupin cultivars, no significant differences in pathogenicity between the different isolates were detected.

These results are consistent with those reported by Cowling *et al.* (1999) although some cultivars were substituted by others of local importance. In both studies *L. angustifolius* cv Wonga was consistently the most resistant and *L. albus* cv Kiev Mutant the most susceptible. Differences in low disease severity indices at some localities on the Cowling set of cultivars could be ascribed to climatic (environmental) conditions.

In both trials, *L. albus* (cv. Kiev Mutant) was most susceptible to the seven isolates followed by the cultivars Swartland, CED 6150, Elsa and Vladimir. *L. angustifolius* cultivars, Wonga and Tanjil were most resistant. In the absence of resistance in sweet *L. albus* cultivars, preferred by the industry, the more resistant *L. angustifolius* cultivars such as Merrit, Wonga and Tanjil are therefore relied upon.

The present study demonstrates significant differences between 16 lupin cultivars in their susceptibility to anthracnose caused by 7 isolates of *C. gloeosporioides* (Table 2.2, 2.3 & 2.4). No significant interaction between isolates and cultivars was detected and no significant differences ($P<0.05$) were noted among isolates in their ability to cause disease on the current set of cultivars used. None of the cultivars displayed inconsistency in terms of their reaction to the seven different isolates of *C. gloeosporioides* collected from the main lupin-growing region of South Africa. All varieties displayed consistent levels of tolerance/resistance in two separate sets of trials to seven different isolates. Tolerance/resistance to anthracnose among the narrow-leafed in *L. angustifolius* (cv. Wonga and Tanjil) was generally higher and showed greater variability compared to *L. albus* (Esta, Kiev Mutant, Vladmir and Hamburg) and *L. luteus* (Juno and Wodjil). This is consistent with the result of Talhinhas *et al.* (1998) where *L. angustifolius* lines showed greater variability in their resistance response to isolates obtained from various parts of the world than *L. albus*. In both trials, the highest level of tolerance/resistance was found in *L. angustifolius* (cv. Tanjil and Wonga) while

*L. albus* (Esta, Kiev Mutant, Vladmir and Humburg) cultivars appeared to be most susceptible (Table 2.2).

Recently J. A. M. van der Mey & D. Fourie (ARC – Grain Crops Institute, Potchefstroom – personal communication) observed unexpected susceptibility under field conditions of one of the lupin lines that tested resistant in the glasshouse. To confirm or reject such observations it is necessary to regularly compare strains of the fungus from different localities and different *Lupinus* spp. for any pathological changes.

The relative levels of resistance among the lupin cultivars observed in our trials are similar to those found in screening trials performed in Australia where resistance was confirmed in glasshouse and field trials for *L. angustifolius* cv Wonga and Tanjil (Cowling *et al.*, 1998). The foliage of these two cultivars was asymptomatic and only minor pod lesions were observed (Cowling *et al.*, 1998). In both trials, Kiev Mutant was most susceptible to the seven isolates followed by Swartland, CED 6150, Elsa and Vladimir. The latter was also reported to be susceptible in three years of field trials in Nova Scotia, Canada (Reddy *et al.*, 1996). Thus, despite the fact that relatively few isolates were tested, the results show that there are no new virulent isolates that can cause damage to the commercially cultivated lupin cultivars Wonga and Tangil. In the short term, the cultivation of existing cultivars should therefore be safe from anthracnose attack in South Africa. Continuous characterization of isolates and monitoring is, however, required in order to avert any potential threats to the local industry.

Cowling *et al.* (1999) reported that field screening for anthracnose resistance is affected by variation in weather and build-up of inoculum during the growing season, which are not relevant while screening 3-week–old seedlings in the glasshouse. Shifts in pathogenicity under field conditions are often not consistent due to the variation in climatic conditions. Reliable results can only be obtained from glasshouse studies as

demonstrated in this study. This method of evaluation also proved to be reliable and consistent in testing different cultivars against different strains of the fungus under glasshouse conditions.

Regular screening of isolates on a "Standard Set of Accessions" in other parts of the world will also aid quarantine authorities to prevent the spread of more virulent strains from one part of the world to another. To ensure that new physiological races of the fungus are not missed, it would be necessary to substitute some of the cultivars in the current sets of differentiating cultivars with more resistant ones as they become available and definite resistance genes are identified.

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**Figure 2.1** Disease severity assessment diagram

1 2 3 4 5

**Table 2.1** Host range, localities and dates from which the seven *C. gloeosporiodes* isolates where collected.

| *C. gloeosporioides* isolates | Host | Locality | Collection Date |
|---|---|---|---|
| SHK 1033 | *L.albus* | Elsenburg near Stellenbosch | 1995 |
| SHK 2140 | *L.albus* cv. CEO | Hopefield | 1999 |
| SHK 2141 | *L.albus* | Roodebloem near Caledon | 1999 |
| SHK 2148 | *L.albus* | Langgewens near Malmesbury | 1999 |
| SHK 2153 | *L.luteus* cv. Laget | Philadelphia | 2000 |
| SHK 2154 | *L.albus* cv.Sonet | Roodebloem near Caledon | 2000 |
| SHK 2155 | *L.albus* cv.Swartland | Roodebloem near Caledon | 2000 |

**Table 2.2** Disease severity expressed by seven *C. gloeosporioides* isolates on 16 *Lupinus* cultivars.

| *Lupinus* cultivars | Disease Severity Indices[1] | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Trial one | | | | | | Trial two | | | | | |
| | SHK1033[3] | SHK2141 | SHK2148 | SHK2140 | Mean | | SHK1033 | SHK2153 | SHK2154 | SHK2155 | Mean | |
| *L. albus* | | | | | | | | | | | | |
| Cedara 6150 | 3.7[4] | 3.4 | 3.6 | 3.8 | 3.6 | | 3.9 | 4.0 | 3.4 | 4.1 | 3.9 | S |
| Esta | 3.7 | 3.8 | 3.7 | 4.0 | 3.8 | S[5] | 4.1 | 4.5 | 2.9 | 4.1 | 3.9 | S |
| Hamburg | 3.5 | 3.4 | 3.9 | 3.3 | 3.5 | | 3.5 | 4.1 | 3.0 | 3.6 | 3.5 | S |
| Kiev Mutant | 3.8 | 4.0 | 4.1 | 4.1 | 4.0 | S | 3.6 | 4.4 | 3.9 | 3.8 | 4.0 | S |
| Swartland | 3.9 | 3.9 | 3.8 | 3.8 | 3.8 | S | 4.3 | 3.9 | 3.6 | 4.1 | 4.0 | S |
| Typtop | 2.7 | 2.4 | 2.3 | 2.7 | 2.5 | | 2.4 | 2.6 | 2.5 | 2.4 | 2.5 | |
| Vladimir | 2.8 | 3.7 | 3.7 | 3.8 | 3.8 | S | 3.8 | 3.7 | 3.5 | 3.9 | 3.7 | S |
| *L. angustifolius* | | | | | | | | | | | | |
| Helderberg | 2.3 | 2.0 | 2.4 | 3.0 | 2.4 | | 2.6 | 3.4 | 2.8 | 2.8 | 2.9 | |
| Merrit | 2.2 | 2.7 | 2.0 | 1.9 | 2.2 | | 1.9 | 2.6 | 1.7 | 2.4 | 2.1 | |
| Moredou | 2.1 | 2.2 | 2.3 | 2.3 | 2.2 | | 1.7 | 2.7 | 1.9 | 1.8 | 2.0 | |
| Sonet | 2.4 | 2.2 | 2.2 | 2.6 | 2.3 | | 2.8 | 2.6 | 2.4 | 2.5 | 2.7 | |
| Tanjil | 1.6 | 1.7 | 2.0 | 1.7 | 1.7 | R | 1.3 | 1.8 | 1.9 | 1.5 | 1.6 | R |
| Wonga | 1.8 | 1.6 | 1.9 | 1.6 | 1.7 | R | 1.4 | 1.9 | 1.3 | 1.7 | 1.6 | R |
| *L. luteus* | | | | | | | | | | | | |
| Juno | 2.4 | 2.0 | 2.4 | 2.4 | 2.2 | | 2.1 | 2.3 | 2.3 | 2.3 | 2.2 | |
| Markiz | 2.3 | 2.0 | 2.4 | 2.4 | 2.3 | | 2.3 | 2.4 | 2.5 | 2.2 | 2.4 | |
| Wodjil | 2.3 | 2.1 | 2.0 | 2.1 | 2.1 | | 2.4 | 2.3 | 2.3 | 1.8 | 2.2 | |
| LSD[2] | 0.500 | 0.484 | 0.457 | 0.654 | 0.259 | | 0.792 | 0.610 | 0.643 | 0.878 | 0.359 | |
| %Cv[6] | 12.6 | 12.6 | 11.5 | 16.3 | 13.4 | | 20.2 | 14.0 | 17.0 | 21.7 | 18.3 | |

[1] Mean disease severity rating on a scale ranging from 0 to 5 where 0 = plants healthy (no symptoms) and 5 = plants dead due to disease.

[2] Fisher's protected t-test least significant difference (LSD) for mean separation at the 5 % level of significance (Snedecor & Cochran, 1980) among cultivars for each isolates.

[3] Accession number for the lupin anthracnose causing *C. gloeosporioides* isolates (Table 1).

[4] Severity value is mean from eight replications.

[5] S = most susceptible groups of cultivars selected with mean rating greater than 3.627 and 2.882, R = Most resistant with mean rating less than 2.125 and 2.049 at P =0.05 for trial one and two respectively using Gupta & Panchapakesan Multiple t-distribution test procedure.

[6] % Coeficient of variation

**Table 2.3** Analysis of variance of anthracnose severity recorded in Trial 1

| Source of variation | DF | SS | MS | F | F Pro |
|---|---|---|---|---|---|
| Isolates | 3 | 0.5571 | 0.1857 | | |
| Isolate replication | 12 | 4.0075 | 0.3340 | 2.42 | |
| Cultivars | 15 | 136.4653 | 10.8977 | 78.83 | <0.001 |
| Isolate x Cultivar | 45 | 6.9214 | 0.1538 | 1.11 | 0.307 ns[1] |
| Residual | 180 | 24.8845 | 0.1382 | | |
| Total | 255 | 199.8358 | | | |

[1]ns = not significant

**Table 2.4** Analysis of variance of anthracnose severity recorded in Trial 2

| Source of variation | DF | SS | MS | F | F Pro |
|---|---|---|---|---|---|
| Isolates | 3 | 6.7086 | 2.2362 | | |
| Isolate replication | 12 | 8.9427 | 0.7452 | 2.81 | |
| Cultivars | 15 | 180.0448 | 12.0030 | 45.23 | <0.001 |
| Isolate x Cultivar | 45 | 14.2881 | 0.3175 | 1.20 | 0.206 ns[1] |
| Residual | 180 | 47.7674 | 0.2654 | | |
| Total | 255 | 257.7517 | | | |

[1]ns = not significant

# CHAPTER 3

# THE PRODUCTION OF GIBBERELLIN-LIKE SUBSTANCES BY *COLLETOTRICHUM GLOEOSPORIOIDES* ASSOCIATED WITH LUPIN ANTHRACNOSE

## ABSTRACT

The most characteristic symptoms of lupin (*Lupinus* spp.) anthracnose caused by the fungus *Colletotrichum gloeosporioides*, are twisting and bending of stems and petioles before definite lesions are observed. These symptoms might be related to the production of plant hormones, such as gibberellin-like substances being produced by the causal organism. In the present study, two isolates of *C. gloeosporioides* (SKH 2148 and SHK 1033) were examined for their ability to produce gibberellin-like substances using the barley bioassay method of Jones & Varner (1967). The isolates were grown in minimal liquid medium for two weeks before the medium was extracted with ethyl acetate following a pH adjustment to 2.5. The ethyl acetate extract was concentrated under vacuum to dryness, and redissolved in acetate buffer (pH 4.8), $CaCl_2$. This solution was then used in the bioassay. Gibberellin-like substance were identified and the concentration of the gibberellin-like substance produced by the fungus was found to be 0.21 μg $GA_3$ equivalents and > 0.26 μg $GA_3$ equivalents per g dry mass of mycelium for isolates SHK 2148 and SHK 1033, respectively. As gibberellins show different modes of action in plants it is therefore of importance to further investigate the role played by gibberellins in this specific pathogen/host interaction

## 3.1 INTRODUCTION

The most common hormones that regulate the growth and physiology of plants are auxins, cytokinins, ethylene, abscisic acid and gibberellins. They act synergistically or antagonistically to regulate growth, but sometimes their effect may simply be additive (Letham *et al.*, 1978). In some cases following infection by a pathogen, specific responses such as change in the morphology of leaves or stems, abnormal root or flower development, and the production of tumours occur. Such responses usually indicate specific interference with growth regulation of the plant by metabolites produced by the pathogen (Andel & Fuchs, 1972).

Gibberellins are a group of natural plant hormones that have various effects on the growth and morphogenesis of plants (Pharis & King, 1985). Gibberellin production has been extensively studied in *Gibberella fujikuroi* (Sawada) Wollenw. (Anamorph: *Fusarium fujikuroi* Nirenburg [G] mating population C in the *G. fujikuroi* species complex), and in cultures of other *Fusarium* species and several bacteria (Andel & Fuchs, 1972). The production of gibberellins in liquid cultures of *G. fugikuroui* was first reported by Kurosawa (1926). Production of gibberellins by *Sphaceloma manihoicola* (Rademacher & Graebe, 1979), *Neurospora crassa* Shear & Dodge (Kawanabe *et al.*, 1983) (as cited by Tudzynski *et al.*, 1998) and more recently by *Phaeosphaeria* spp. Cesati & de Notaris L487 (Kawaide & Sassa, 1993) has been reported.

Lupin anthracnose caused by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Weimer, 1943; Sweetingham *et al.*, 1995; Reddy *et al.*, 1996) is a devastating disease (Ageav, 1993; Gondran, 1994; Savchenko *et al.*, 1994; Paultiz *et al.*, 1995;Sweetingham *et al.*, 1995; Frencel, 1998; Baer *et al.*, 1999). Despite the seriousness of the disease, biochemical and physiological aspects involved in the infection process have not been investigated to any significant degree. The main

symptoms of the disease are lupin plants displaying bent and twisted stems and dark brown, elongated lesions in the crook of the bend (Gondran, 1994; Murray, 1996; 1997). Diseased stems collapse, displaying characteristic tissue necrosis and crook-shaped distortion (Frencel, 1998). Lesions also develop on leaves and pods (Murray, 1996). This phenomenon prompted us to investigate the possibility of a growth hormone being involved in the host-pathogen interaction. In other words, whether distortions appear before lesion formation and not due to collapse and dying associated with necrosis. The objective of the present study was therefore to ascertain whether *C. gloeosporioides* associated with lupin anthracnose produces a gibberellin-like substance *in vitro*.

## 3.2 MATERIALS AND METHODS

To determine whether the *Colletotrichum* isolates associated with lupin anthracnose produce gibberellin substances, the bioassay described by Jones & Varner (1967) was used with little modification. The conversion factors (CF) for the starch sample were first determined at 20 °C and 25 °C respectively by using the following equation:

$$CF = \mu g\ \alpha\text{-amylase} \times t \times v / \Delta OD. \times T_V$$

Where $T_V$ = total volume of supernatant, $\Delta$O.D = OD of zero time (control) - OD of reaction sample, CF = conversion factor for starch sample, t = time of incubation and v = volume of supernatant taken for incubation. Then the relationship (standard curve) between gibberellic acid ($GA_3$) concentrations and $\alpha$-amylase released from barley half-seeds used in this study was determined. Only then was the concentration of gibberillin-like substances produced by the test fungi determined.

### 3.2.1 Gibberellin extraction process

Two single-spore isolates of *C. gloeosporioides* (SHK 1033 and SHK 2148) were grown for ten days in liquid minimal medium (2g $NaNO_3$, 30 g sucrose, 1g $KH_2PO_4$, 0.5 g $MgSO_4 \cdot 7H_2O$, 0.5 g KCl, 1000ml distilled $H_2O$ supplemented with 0.2 ml trace element solution in 250 ml Erlenmeyer flasks. Ten flasks containing 150 ml the medium was used for each isolate. One flask from SHK 1033 was rendered void due to contamination. The trace element solution consisted of 5 g citric acid, 5 g $ZnSO_4$, 1 g $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 250 mg $CuSO_4.5H_2O$, 50 mg $MnSO_4$, 50 mg boric acid, 50 mg $Na_2MoO_4 \cdot 2H_2O$ and 95 ml distilled water. Cultures were continuously shaken except for the first day after inoculation of flasks. After 10 days, the mycelial mat was harvested on Whatman Nº 1 filter paper and the filtrate retained. The harvested mycelium was dried at 70 $^0$ C for 48 h and weighed. The pH of the filtrate was adjusted to 2.5 using 1 N HCl. The gibberellin-like substance was extracted with ethyl acetate from the filtrate using a separation funnel. The ethyl acetate fraction was dried using anhydrous $Na_2SO_4$ and concentrated at 45 $^0$C using a Buchi Rotavapor-R under vacuum. The ethyl acetate fraction was then subsequently dissolved in an acetate buffer solution (50 ml of 3.8 mM acetic acid adjusted to 4.8 pH using 4 mM sodium acetate solution) to a final volume of 4 ml. The solution was centrifuged for 10 min. at 2000g x at 4 °C. This solution was then used in the bioassay to detect gibberellin-like substances (Groenewald & Grobbelaar, 1978)

### 3.2.2 α-Amylase assay

A starch solution for the α-amylase assay was prepared from 150 mg of potato soluble starch (Sigma S2630), 600 mg $KH_2PO_4$ and 3 mg calcium chloride in a total volume of 100 ml distilled water. The mixture was boiled for 1 min and then cooled before the clear supernatant was decanted off and used for the assay. An iodine stock

solution was prepared by mixing 6 g of potassium iodide and 600 mg of iodine in 100 ml of sterile distilled $H_2O$. One ml of the stock solution was added to 0.05 N hydrochloric acid to give a final volume of 100 ml. This was used to stop the α-amylase reaction.

Two-year-old barley seed (*Hordeum vulgare L.* cv. Chariot garssand) was dehusked by treating with 50% sulphuric acid for 1 hr. The acid was decanted and the seed was washed thoroughly in distilled $H_2O$ and air-dried overnight (Reeve & Crozier, 1975). Seeds were cut in half transversely and the embryo portion discarded. The halved endosperms were disinfected by soaking in 1% NaOCl for twenty minutes, followed by three consecutive rinses in sterile $H_2O$. Disinfected seeds were imbibed on sterile sand in 100 mm glass Petri dishes each containing 100 g sand, moistened with 20 ml sterile $H_2O$ and placed in incubator at 25 °C. After a 3-d imbibing period, ten halved-seeds were transferred to 25 ml Erlenmeyer flasks containing 0.5 ml of acetate buffer (pH 4.8), 0.2 ml of 132 mM of $CaCl_2$, and 0.2 ml of the standard $GA_3$ solution or culture filtrate in a final volume of 2 ml with distilled $H_2O$. Distilled $H_2O$ was added in control treatment instead of the filtrate. As an added safety measure against microbial contamination, 0.2 ml of 310 µM chloramphenicol was added to each flask. Flasks containing the $CaCl_2$ and buffer were autoclaved prior to the start of the incubation period. Incubation of the halved seeds in the test solution was continued for 24 hours at room temperature with continuous shaking at 40 oscillations/minute. Following incubation, the contents were decanted into 100 x 7.5 mm centrifuge tubes and flask was three times thoroughly washed, each time with 1 ml of distilled $H_2O$. The washing was added to respective tube. The tubes were then centrifuged for 10 minutes at 2000 x g at 4 $^0$C. Clear supernatant was decanted off and used for the assay.

The assay for α-amylase was accomplished using suitable volumes (0.02 –0.2 ml) of the supernatant fraction of 5-µg/ml α-amylase and sufficient water to make a total

volume of 1.0 ml. The chemical reaction was started by adding 1.0 ml of starch solution or suspension to the medium and allowed to continue for a suitable period of time (1-10 min). The reaction was stopped by the addition of 1.0 ml of iodine reagent. To this final reaction mixture, 5.0 ml of distilled $H_2O$ was added, shaken and the optical density (OD) determined at 620 nm by means of a Spectronic 20 spectrophotometer. OD values were converted to the amount (μg) of α-amylase released, using a factor that was obtained for the particular starch sample by incubation with pure α-amylase. The concentration of α-amylase was calculated according to the following equation: α-amylase (μg) = ΔO.D. X $T_V$ X CF / t X v.

### 3.3 RESULTS

The incubation time was determined using the pure α-amylase assay. According to Jones & Varner (1967), the decrease in OD at 620 nm is directly proportional to the quantity of α-amylase present in the reaction mixture. The most accurate result is obtained by adjusting the α-amylase enzyme aliquot and time (t) so that the optical density of the starch-iodine complex is about 50% that of the zero time control. Based on this, the incubation period for the trials was set to be 3 minutes at 20 $^0$C and 5 minutes at 25 $^0$C for the two trials (Table 3.1).

The amount of α-amylase produced by different concentrations of $GA_3$ is presented in Fig. 3.1. A nearly straight line was obtained that could be used as a Standard Curve to determine the amount of gibberellin-like substances produced by the fungal isolates.

Both fungal filtrates activated the secretion of α-amylase in halved barley seed (Table 3.2). Gibberellin-like secretions were determined to be 0.22 μg and >0.26 μg $GA_3$ equivalents per g dry mass of mycelium for isolates SHK 2148 and SHK 1033,

respectively (Table 3.3). However, a very small amount of α-amylase was produced in the control treatments.

## 3.4 DISCUSSION

The quantification of α-amylase released from barley half-seeds provided a suitable method for the determination of gibberellin-like substances in liquid culture (Jones & Varner, 1967). This is despite the fact that not all barley cultivars are suitable for use as there is considerable variation among varieties in their sensitivity to $GA_3$. In addition, sensitivity is associated with season, area of growth and age of the seed. The barley aleurone bioassay is sensitive to $GA_3$ in doses as little as 0.001 μg per flask (Reeve & Crozier, 1975) or even $9 \times 10^{-12}$ g (9 pg) of GA3 as mentioned in Salisbury & Ross (1992). The barley aleurone layer is well established as a model for studying hormonal regulation (as cited by Hamabata *et al.*, 1994). A characteristic of the aleurone layer of cereal seeds is its capacity to secrete a wide spectrum of hydrolytic enzymes, which play a role in endosperm degradation during germination (Moll & Jones, 1982). In barley aleurone, the synthesis of many enzymes is controlled by gibberellin, calcium and abscisic acid (Hillmer *et al.*, 1992). $GA_3$ induces the synthesis of α-amylase and several other hydrolytic enzymes (Ho, 1991; Jacobsen & Close, 1991). Protoplasts isolated from aluerone layers of barley responded to externally applied gibberellic acid with increased synthesis and the secretion of α-amylase (Gilroy & Jones, 1994). The quantity of α-amylase produced is proportional to the logarithm of gibberellin concentration applied (Jones & Varner, 1967).

Cytochemical and metabolic evidence has shown that the release of α-amylase from the halved barley is dependent on metabolic linked processes and not diffusion limited (Moll & Jones, 1982). Heat shock imposed by raising the temperature of

incubation from 25°C to 40°, inhibited or suppressed the accumulation of α-amylase and other proteins in the incubation medium of barley aleurone layers treated with GA and $Ca^{+2}$ (Brodl *et al.*, 1990; Sticher *et al.*, 1990; Lanciloti *et al.*, 1996). Different responses in terms of α-amylase released from the two trials in this experiment were obtained because they were made at two different temperatures. The concentration of α-amylase released from incubation at 25 $^0$ C was higher compared to that released from incubation at 20 $^o$ C (Table 3.2). This finding is consistent with a report by Agu & Palmer (1997) where a malting temperature of 30°C produced more α- amylase than one of 20°C.

Despite great care being taken to eliminate $GA_3$ already present in the husk of the seed using sulphuric acid, very small amounts of α-amylase were still produced in the control treatment. Some reports associate α-amylase production with acidification of the starchy endosperm by aleurone cells, but in the present experiment, the pH of the incubation medium was not monitored. Hambata *et al.* (1988) and Macnicol & Jacobsen (1992) demonstrated that acidification of the endosperm was produced by the aleurone cells *per se* independent of the embryo or gibberellin present. Sinjorgo *et al.* (1993) reported increased activity of α-amylase and glyoxysomal isocitrate lyase (ICL) independent of gibberellic acid at pH 3.2, although the effect was most pronounced for ICL. At higher extracellular pH values, hormone induced enzyme activity decreased in a dose-dependent manner.

Some evidence is available that $GA_3$-producing microorganisms often induce harmful reactions in host plants. In isolates of *F. fujikuroi*, the causal agent of *bakanae* disease of rice, a positive correlation existed between the biosynthesis of $GA_3$ *in vitro* and symptom expression (Sunder *et al.*, 1998). In *Sorghum bicolor* (L.) Moench, the application of $GA_3$ reversed floral primordials to vegetative leafy growth, or phyllody, a common symptom associated with sorghum head smut. It was concluded that phyllody

in partially infected sorghum plants could be due to an increase in $GA_3$ in affected plants (Bhaskaran *et al.*, 1990). Gibberellin found in the extract of the fungal culture medium promoted shoot elongation in sorghum (Beall *et al.*, 1991). Matheussen *et al.* (1991) noted a reduction in early-13-hydroxylation precursors in all the smutted or sterile panicle samples compared to their appropriate controls. This reduction suggests that this pathway is blocked or that precursors are diverted to other uses by the fungus (*Sporisorium reilianum*), although the fungus can produce $GA_1$ and $GA_3$ when cultured on Richard's medium. The result could be due to a deficiency of bioactive gibberellins at the time of internode elongation

Reports also indicate that exogenously applied abscisic acid or gibberellic acid increased the susceptibility of non-host plants to cowpea rust (Li & Heath, 1990a). Intercellular washing fluids obtained from rust-infected bean (*Phaseolus vulgaris* L.) leaves and other compatible rust-plant interactions also suppressed silica deposition in bean and increased the incidence of haustoria subsequently produced by the cowpea rust fungus (Heath, 1979; 1981). In contrast, however, Li & Heath (1990b) did not see any effect using intercellular washing. In cowpea rust, the absence of haustoria in non-host interactions is associated with the presence of silica deposits on the plant wall (Heath, 1979; Stumpf & Heath, 1985). Earlier, Soni *et al.* (1972) reported that silica deposition is regulated by gibberellic acid in *Avena* epidermal cells. Injection of both abscisic acid and gibberellic acid resulted in decreased silica deposition and autofluorescent walls in mesophyll cells (Stumpf & Heath, 1985; Li & Heath, 1990a) and increased the incidence of haustoria (Li & Heath, 1990a). A similar effect was also observed for the corn rust fungus, except that the incidence of autofluorescent walls was not reduced, probably because of the increased autofluorescence of the contents of invaded cells (Li & Heath, 1990a).

The present study represents the first time that the production of Gibberellin-like substances has been associated with any *Colletotrichum* spp. Preliminary results of the present study suggest that the detection of gibberellin-like substances from pure cultures of *C. gloeosporioides* grown in minimal liquid medium could pave the way for assessing its effect in host pathogenesis. Bending and twisting of the stems and petioles, and deformation of pods and leaves of lupins infected by *C. gloeosporioides* even before lesions are visible, is probably related to $GA_3$ production of the pathogen as gibberellins stimulates cell division, promotes cell growth and cell-wall plasticity (Salisbury & Ross, 1992). Furthermore, Salisbury & Ross (1992) speculated that the sugars released in the host plant by hydrolase enzymes induced by gibberellins might serve as energy source for the fungus.

Sunder *et al.* (1998) suggested that prior knowledge of the ability of *F. fujikoroi* isolates from *bakanae* disease of rice to produce more $GA_3$ may help in selecting isolates for testing disease resistance. Similarly, if increased $GA_3$ production in lupins can be correlated with increased severity of symptoms in the *C. gloeosporioides*–lupin interaction, this knowledge may be helpful in screening cultivars for anthracnose resistance provided that the association is established experimentally. It is therefore important that the role gibberellins play lupin anthracnose is further investigated.

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**Table 3.1** Pure α-amylase assay for the determination of the conversion factor (CF) from starch samples at incubation temperatures of 20 and 25 $^{0}$C for 5 min and 3 min, respectively

| 20 $^0$C | | | 25 $^0$C | | |
|---|---|---|---|---|---|
| Zero OD (0 min) | Final OD (5 min) | CF[1] | Zero OD (0 min) | Final OD (3 min) | CF |
| 0.70 | 0.28 | 0.30 | 0.95 | 0.44 | 0.15 |
| 0.75 | 0.32 | 0.29 | 0.90 | 0.38 | 0.14 |
| 0.70 | 0.29 | 0.30 | 0.95 | 0.37 | 0.13 |
| 0.75 | 0.27 | 0.26 | 0.95 | 0.48 | 0.16 |
| 0.75 | 0.28 | 0.27 | 0.90 | 0.38 | 0.14 |
| 0.70 | 0.36 | 0.37 | | | |
| 0.70 | 0.32 | 0.33 | | | |
| 0.70 | 0.30 | 0.31 | | | |
| Average | | 0.30 | | | 0.14 |

Note : 0.2 ml α-amylase (supernatant) at a concentration of 5 µg/ml and 8 ml of $T_V$ was used for assaying

[1]Conversion factor (CF) = µg α-amylase X t X v / ΔOD. X $T_V$ where $T_V$ = total volume of supernatant, ΔO.D = OD of zero time (control) - OD of reaction sample, C.F. = conversion factor for starch sample, t = time of incubation and v = volume of supernatant taken for incubation

[2] Optical density reading at zero time control

[3] Optical density reading following incubation

[4] Incubation periods in minutes.

**Table 3.2** Average change in optical density (OD) following the effect of $GA_3$-concentration on α-amylase released by barley seed incubated at 20 $^0$C and 25 $^0$C for 5 min and 3 min, respectively.

| | 20 $^0$ C | | | | | 25 $^0$C | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatment | TVS[1] | Zero OD[2] | Final OD[3] | Δ OD[4] | α-amylase[5] (μg) | TVS | Zero OD | Final OD | Δ OD | α-amylase[5] (μg) |
| 5 x $10^{-5}$ μg $GA_3$ | 5.2 | 0.60 | 0.45 | 0.15 | 0.23 | 5.0 | 0.79 | 0.74 | 0.05 | 0.06 |
| 5 x $10^{-4}$ μg $GA_3$ | 5.4 | 0.52 | 0.52 | 0.11 | 0.10 | 5.0 | 0.78 | 0.69 | 0.09 | 0.11 |
| 5 x $10^{-3}$ μg $GA_3$ | 6.1 | 0.43 | 0.43 | 0.11 | 0.19 | 6.1 | 0.76 | 0.59 | 0.17 | 0.25 |
| 5 x $10^{-2}$ μg $GA_3$ | 3.5 | 0.22 | 0.22 | 0.05 | 0.10 | 6.2 | 0.75 | 0.47 | 0.28 | 0.42 |
| 5 x $10^{-1}$ μg $GA_3$ | 5.4 | 0.39 | 0.39 | 0.19 | 0.31 | 5.4 | 0.76 | 0.42 | 0.34 | 0.44 |
| SHK 2148 | 5.3 | 0.31 | 0.31 | 0.20 | 0.32 | 5.0 | 0.71 | 0.32 | 0.39 | 0.47 |
| SHK 1033 | 5.3 | 0.26 | 0.26 | 0.21 | 0.33 | 5.8 | 0.73 | 0.29 | 0.44 | 0.61 |
| Control | 5.2 | 0.675 | 0.64 | 0.04 | 0.06 | 5.5 | 0.94 | 0.94 | 0.01 | 0.03 |

Note: Conversion factor 0.30 and 0.14 were used to calculated α-amylase level produced at 20 $^0$ C and 25 $^0$ C for 5 and 3 min. incubation period respectively; 0.2 ml was the volume of supernatant taken for incubation; [1]Total volume of supernatant; [2] Optical density reading at zero time control; [3] Optical density reading following incubation; [4] The difference between optical density reading at zero time control and optical density reading following incubation; [5]α-amylase (μg) = ΔO.D. X $T_V$ X CF / t X v.

**Table 3.3** Total dry mass of mycelium (g), average μg α-amylase produced, and the extrapolated average $GA_3$-equivalents (μg/g) of dry mycelium for two isolates of *C. gloeosporioides*

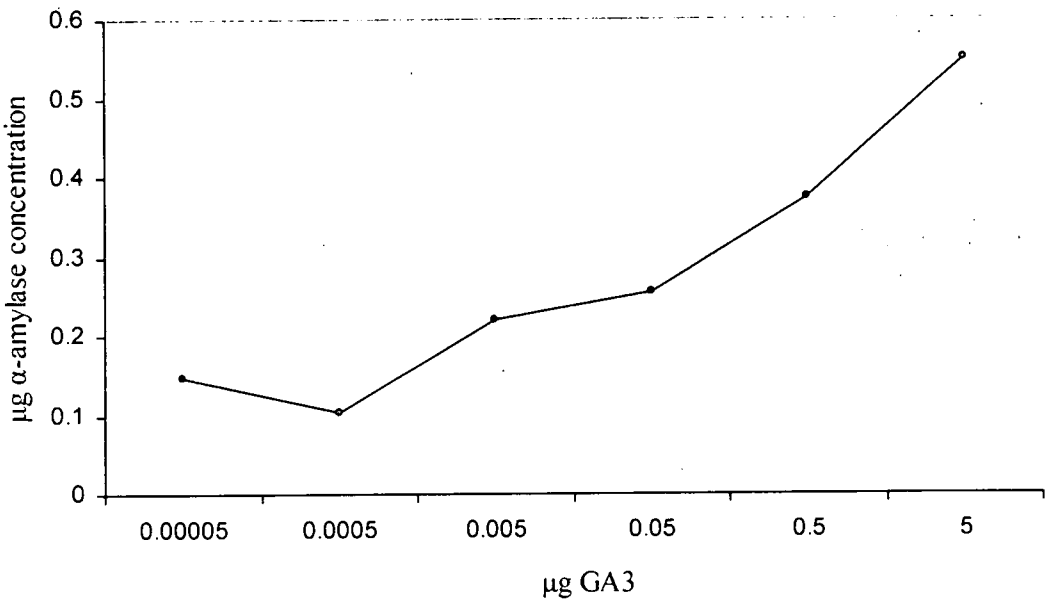
| Isolate No. | TDMW[1] (g) | LMM[2] (l) | α-amylase (μg) | $GA_3$-like substance (μg) | $GA_3$ equivalent (μg)/ g mycelial dry mass |
|---|---|---|---|---|---|
| SHK 2148[3] | 23.13 | 1.35 | 0.40 | 0.500 | 0.22 |
| SHK 1033 | 19.18 | 1.50 | 0.46 | >0.500 | >0.26 |
| Control | 0.00 | 1.50 | 0.04 | < 0.005 | |

[1] Total dry mycelium weight

[2] Liquid minimal medium used in liters

3 Derived from Standard Curve (Fig 1)

**Figure 3.1** Relationship between gibberellic acid ($GA_3$) concentration and α-amylase release from barley half-seeds (standard curve)


0.6
0.5
0.4
0.3
0.2
0.1
0
µg α-amylase concentration
0.00005
0.0005
0.005
0.05
0.5
5
µg GA3

# CHAPTER 4

# INFECTION PROCESS OF *C. GLOEOSPORIOIDES* ASSOCIATED WITH LUPIN ANTHRACNOSE ON TWO DIFFERENT *LUPINUS* SPP. VARYING IN SUSCEPTIBILITY

## ABSTRACT

Understanding the mechanisms of infection can be helpful in the development of strategies for controlling anthracnose of lupin caused by *C. gloeosporioides*. An isolate of *C. gloeosporioides* was studied using fluorescence (light) and scanning electron microscopy to compare pre- and post-penetration events at various times after artificial inoculation of lupin leaves. Qualitative studies of germination and the penetration process showed that processes were similar in susceptible and less susceptible hosts. Conidia begin to germinate at 6 h post inoculation (hpi). Only single, sessile, or subtended appressoria were formed at the tip of the germinating conidium or germtube. Penetration of leaf cells seemed to occur by means of an appressorium and infection peg. By 96 hpi, small spherical vesicles and primary hyphae were present in the penetrated epidermal cell. There was initially, however no extensive development of hyphae inside penetrated epidermis cells.

## 4.1 INTRODUCTION

Anthracnose of lupin (*Lupinus* spp.) caused by *Colletotrichum gloeosporioides* (Penz.) Penz & Sacc. (Sweetingham *et al.*, 1995) is a devastating disease wherever lupins are grown [Chile (Baer *et al.*, 1999); North America (Paultiz *et al.*, 1995); Europe (Gondra, 1994, Frencel, 1998); Russia (Ageav, 1993; Savchenko *et al.*, 1994); Australia (Sweetingham *et al.*, 1995); South Africa (Koch, 1996) and New Zealand (Dick, 1994)]. All aboveground parts of the plant are affected at all the growth stages and one of the most characteristic symptoms is the curling or twisting of stems and petioles. The disease is mainly spread by seed and it is believed that one infected seed in 10 000 seeds can lead to an epidemic within two years. The disease causing organism is disseminated by water splash, debris and vehicle, animal or human movement.

Factors that affect the success of the host pathogen interaction are mainly virulence of the pathogen, host susceptibility and climatic conditions (Agrios, 1988). Differences in susceptibility towards the disease within the genus *Lupinus* were recorded (Sweetingham, 1997; Cowling *et al.*, 1999; Chapter 2) and disease development was found to be best between 18-24 °C under high humidity conditions (Sweetingham, 1997).

Conidial attachment, germination, appressorial formation, and the formation of infection hyphae are independent processes as reviewed in Chapter 1. Although the infection processes within *C. gloeosporioides* on different hosts were widely studied (Binyamini & Schiffmann-Nadel, 1972; Brown 1975; Brown, 1977; TeBeest *et al.*, 1978; Chau & Alvarez, 1983; Vinijsanun *et al.*, 1987; Wei *et al.*, 1997) it is believed that the lupin/*C. gloeosporioides* interaction is unique although some general similarities might exists. The confusion in the identity of the pathogen (Gondran, 1994; Reed *et al.*, 1996;

Koch, 1996; Yang & Sweetingham, 1998; Lardner *et al.*, 1999; Johnston, 2000) is one reason why we believe the interaction to be unique.

In this study early host/pathogen interactions were studied on a microscopic level. The early stages of recognition and infection by the pathogen on two *Lupinus* spp. varying in susceptibility under glasshouse conditions were followed.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Inoculum production and artificial inoculation of plants

Inoculum was obtained from 7-d-old cultures of *C. gloeosporioides* [SHK 1033-Koch (1996)] grown on potato dextrose agar (PDA)(4 g potato extract, 20 g dextrose and 15 g agar per one liter of distilled water ($dH_2O$) supplemented with streptomycin (0.3 ml/l). Conidia were harvested by washing the surface of the agar with sterile $dH_2O$ and filtering through cheesecloth to remove mycelium. The density of the conidial suspension was determined using a haemocytometer and adjusted to $5 \times 10^5$ conidia $ml^{-1}$ with sterile $dH_2O$. One drop of Tween-20 was added to 500 ml conidial suspension.

Seed of *Lupinus albus* cv Kiev Mutant (susceptible) and *Lupinus angustifolius* cv Wonga were disinfected with a 1 % NaOCl solution. Seed was planted in 1:1 ratio of virgin loam soil and perlite (Genolite). Seedlings were maintained in glasshouse at 20 °C ± 3 °C prior to inoculation. Fourteen-day-old seedlings in the 3rd leaf stage were sprayed with the inoculum suspension until run-off. A spray gun at 1.8 KPa air pressure was used to apply inoculum. Inoculated seedlings were placed in a dark moist chamber for 24 hrs. At respectively 6, 8, 12, 16, 24, 48, 72, 96, 120 and 144 hrs after inoculation, leaf pieces 2-6 mm X 2-6 $mm^2$ in size were cut at random from the plant and for electron microscopy and

fluorescence microscopy immediately fixed with 4% gluteraldehyde in 0.1 M phosphate buffer (30.5 ml of 0.2 M $Na_2HPO_4$, 19.5 ml 0.2 M $NaH_2PO_4$ in 50 ml of $dH_2O$). For light microscopy, stem and leaf pieces were used. The epidermis was torn from the pieces and directly mounted in a lactophenol cotton blue solution. Lactophenol cotton blue solution was prepared by dissolving 20 g phenol crystals in 20 ml water over heat. Lactic acid (20 g) and glycerol (40 g) were added to the phenol solution. Cotton blue (0.5 g) was added to 100 ml lactophenol solution. The experiment was repeated twice.

### 4.2.2 Scanning electron microscopy

Leaf tissues fixed in 4% v/v gluteraldehyde in 0.1 M phosphate buffer were washed in phosphate buffer before they were fixed in 1% w/v $OsO_4$ in 0.1 M phosphate buffer for 30 minutes, followed by an additional washing in. phosphate buffer. Specimens were dehydrated in a series of 50%, 70%, and 95% v/v acetone for 30 minutes per step. Specimens were left in 100% acetone for 24 hrs prior to a final 100% acetone dehydration for 2 hrs before critical point drying in a Polaron E 3000 critical point dryer. The dried specimens were mounted on aluminium stubs using double-sided tape and coated with gold in a Bio-Rad sputter coat machine at 18 mA and 6 x $10^{-1}$ mbar for 135 seconds. Observations were made at 5-10 kV using a JOEL JSM-6400 scanning electron microscope.

### 4.2.3 Fluorescence microscopy

Leaf specimens were cleared and fixed in ethanol-dichloromethane (3:1 v/v) solution supplemented 0.15 % v/v tricloroacetic acid for at least 18-24 hrs. Specimens were washed twice with 50% ethanol for 15 minutes. Additional washing was done in 0.05 M NaOH (2 x 15 min) followed by three rinses with $dH_2O$. Uvitex (0.1%) was prepared by adding 0.8g

130% Uvitex in 100 ml pH 5.8 Tris buffer (6.05 g 0.1 M Tris base in 300 ml $dH_2O$, pH is then adjusted with HCL to 5.8, then make it up to 500 with $dH_2O$). Before specimens were stained with 0.1% Uvitex (Uvitex 2BT 130%) in Tris HCL buffer pH 5.8 solution for 5 min, they were soaked in Tris/HCL buffer pH 5.8 for 30 min and then thoroughly washed 4 times with $dH_2O$. Finally, specimens were soaked in 25% w/v aqueous glycerol for 30 min and then stored in 50 % w/v glycerol solution containing a trace of lactophenol to prevent deterioration of the fungus, and dessication of plant material (Bender *et al.*, 2000).

## 4.3 RESULTS AND DISCUSSION

Leaf wetness was difficult to obtain especially on the adaxial side of leaves that could be ascribed to the absence of trichomes and why conidia did not readily attach to this side of the leaves. Other surface factors could also have played a role. Conidia were also found not to attach to the hypocotyls in contrast with beans (*Phaseolus vulgaris* L.) where *C. lindemuthianum* readily attach to the hypocotyl. John Bailey & co-workers (Clevedon, UK - personal communication) observed the same response. Conidia showed a tendency to accumulate in the trichome cavity and around the trichomes as reported by Bandounas *et al.* (1999). Trichomes have a broad base and a needle like cymbiform appearance. They also found that some of the conidia that germinate on the trichomes produce long germtubes that traveled along the trichome and only once it reached the epidermis produced appressoria. Direct penetration of trichome base as described by TeBeest *et al.* (1978) was not observed. Koch (unpublished data) observed that conidia within the trichome cavities and in suspension on glass slides readily produced condia at the end of germtubes. Baxter *et al.* (1982) reported the same phenomena in *C. acutatum* in slide culture. This aspect needs

further investigation because it can influence the dynamics of the disease through increased inoculum production in a suitable microclimate.

Adhesion of conidia takes place within 6 hours past inoculation (hpi) and conidia began to germinate by producing a single germtube towards one end of the conidium (Fig.4.1). Often germtubes were produced at both ends of the conidia (Bandounas *et. al.*, 1999). A single septum develops within the germinating conidia (Fig.4.1 and Fig.4.5B). No special orientation in growth of the germtubes was observed.

Within 24 hpi, melanized appressoria were formed. A single sessile or subtended appressorium was formed close to the end of the conidium or on the tip of the germtube, respectively. The appressoria appeared to be delineated by a septum from the germtube or conidia (Fig.4.2) and were produced indiscriminately on the surface of the leaves, irrespective of the leaf topography.

Forty-eight hpi infection pegs in the appressoria started to develop and were clearly visible under the fluorescence microscope (Fig.4.4). It was evident that penetration started between 48 and 72 hpi as appressoria started to shrink in both susceptible and resistant interactions (Fig. 4 3) and by 96 hpi had collapsed completely (Fig.4.5).

Similarly, Morin *et al.* (1996) reported the melanization of appressoria of *C. gloeosporioides f. sp. malvae* was evident on all plants examined regardless of the host species. However, after successful penetration of an epidermal cell, development and production of secondary hypha was extensive on the susceptible host. In moderately resistant hosts, colonization was stopped by hypersensitive reaction of the cells adjacent to initial infection sites and no secondary hyphae were produced (Morin *et al.*, 1996). Although there is a time delay, this observation is consistent with earlier reports that penetration of epidermal cells of *Sylosnthes scabra* (Trevorrow *et al.*, 1988) and avocado

fruit (Binyamini & Schiffmann-Nadel, 1972) by *C. gloeosporioides* occurred directly by means of fine infection hyphae. Penetration occurred thought the cuticle and epidermal cell walls as was reported in other *C. gloeosporioides*-host interactions studied previously (Chau & Alvarez, 1983; Morin *et al.*, 1996). Stomatal pores were not the sites of entry, a fact consistent with earlier reports that appressoria of *C. gloeosporioides* were rarely observed in stomatal cavities (Brown 1975; Chau & Alvarez, 1983) and penetration through stomatal pores was rarely observed (TeBeest *et al.*, 1978; Morin *et al.*, 1996).

After 72 hpi, appressoria started to shrink (Fig. 4.3) and after 96 hpi appressoria had completely collapsed (Fig.4.5). By this time small spherical vesicles and primary thin hyphae were present in the penetrated epidermal cell (Fig.4.6). In other case studies one or several intracellular primary hypha are reported emerging from vesicles (Morin *et al.*, 1996; Wei *et al.*, 1997). Although single primary hyphae had begun to develop, there was no extensive development of hyphae inside the first infected epidermal cell (Fig.4.6). Any attempts to follow the infection process further were to no avail. Trevorrow *et al.*, (1988) and Morin *et al.* (1996) reported intracellular infection vesicles within epidermal cells, while no subcuticular colonization was seen in a *C. gloeosporioides f. sp. malvae*-Malvaceae (*Malva parviflora L.*) interaction (Morin *et al.*, 1996). In contrast, infection vesicles located under the cuticle are reported in the interaction between *C. gloeosporioides f. sp. guiansense* and *S. guianensis* (Vinijsanun *et al.*, 1987) while Irwin *et al.* (1984) reported development of a network of profusely branching subcuticular hyphae of *C. gloeosporioides* on *Stylosanthes* spp.

In compatible and incompatible interactions between *C. gloeosporioides f. sp. guiansense* and *Stylosanthes scabra*, Trevorrow *et al.* (1988) reported a significantly higher penetration levels rate for compatible interactions. However, up to the infection vesicle

formation stage (96 hpi), there were no apparent qualitative differences between compatible and incompatible combinations (Trevorrow *et al.*, 1988). In this study, no differences between the initial adhesion, germination, appressoria formation and early infection between the susceptible *L. albus* cv Kiev Mutant and *L. angustifolius* cv Wonga were observed.

Furthermore, Trevorrow *et al.* (1988) showed that in incompatible interactions between *C. gloeosporioides f. sp. guiansense* and *S. scabra*, hyphae remained confined to the epidermis cells. However, in the compatible interaction, the fungus had colonized the mesophyll and ultrastructural studies showed extensive cell damage by that time. In the present study, the period before 96 hpi did not seem critical to the establishment of susceptible or less susceptible response in the host. In both susceptible and less susceptible reactions, the pathogen is still restricted to a single epidermal cell. Attempts to monitor the development of the pathogen after the production of primary hyphae was to no avail. Whether post initial infection peg formation interactions between lupin and *C. gloeosporioides* conform to descriptions of the compatible and incompatible interactions previously studied (Trevorrow *et al.*, 1988; Morin *et al.*, 1996) is open to question.

Our results suggest that the resistance of lupin to anthracnose may be related to fungal development after penetration rather than establishment. Although this does seem to be consistent with earlier reports that pathogen compatibility or incompatibility was unlikely to appear during penetration and vesicle formation (Irwin *et al.*, 1984; Morin *et al.*, 1996), the resistance process still warrants further investigation.

In the present study a typical biotrophic relationship is shown, where the infection hyphae enters the lumen of the host epidermal cell and swells to form an infection vesicle and limited primary hyphae. The inability to follow the infection further in both susceptible

and less susceptible host plants might indicate that the fungus is able to regulate further development and avoid initial cell death. During this period, the fungus probably creates a cellular environment suitable for rapid fungal growth during later stages (necrotrophic) of the disease.

In Chapter 3 it was shown that the fungus produces gibberellin-like substances *in vitro*. Mechanisms of gibberillin actions as listed by Salisbury & Ross, (1992) in broad are stimulation of cell division in shoot apex, promotion of cell growth by increased hydrolysis of starch, fructans and sucrose into glucose and fructose molecules and increase in cell-wall plasticity. They also speculated that in the case of *Gibberella fujikuroi* (Sawada) gibberillins might enhance hydrolysis of starch to sugars in host plants by inducing formation of amylase enzymes, thereby obtaining a sugar food source. Sunder *et al.* (1998) showed that a positive correlation exists between biosynthesis of $GA_3$ *in vitro* and pathogenic behaviour *G. fujikuroi* (*Fusarium moniliforme*) on rice.

We speculate that the lupin pathogen produces gibberellins that hydrolyze complex sugars in both susceptible and less susceptible interactions with the host after penetration and during the biotrophic phase. These simple sugars are then used by the fungus as a food source for further colonization of the host. The production of gibberellins might also be responsible in loosening cell walls and growth promotion by diffusing to parts not directly in contact with the fungus. The first hypothesis would apply to the biotrophic phase of the infection, which is followed by a necrotrophic phase and the second hypothesis to the curling of the stems and petioles before necrotic lesions are visible. If these hypotheses prove to be true it would be the first time that interactions of this nature between a fungal pathogen and plant has been shown since the identification of gibberellins.

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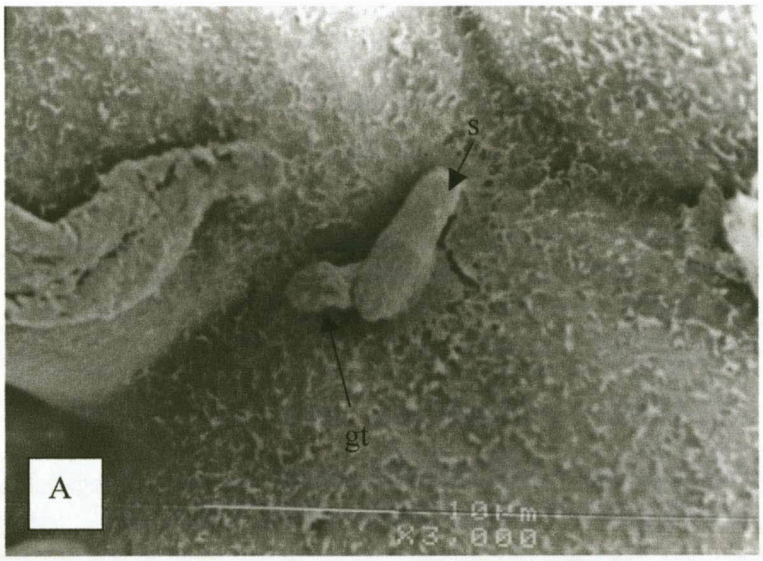
**Figure 4.1** Germinating conidia of *C. gloeosporioides* at 6 hpi on susceptible (A) (Bar = 10μm, 3,000 x) and less susceptible (B) (Bar = 10μm, 2,500 x) lupin leaves: Spore (s); germtube (gt); and septum (sp).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A
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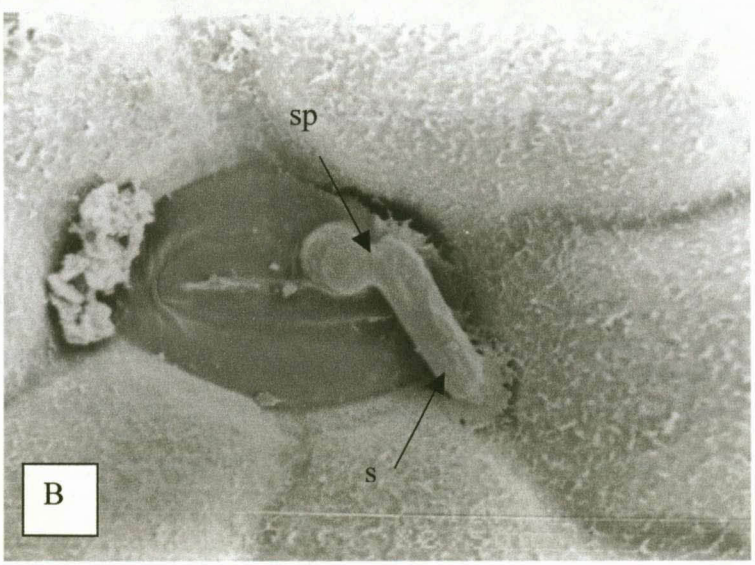
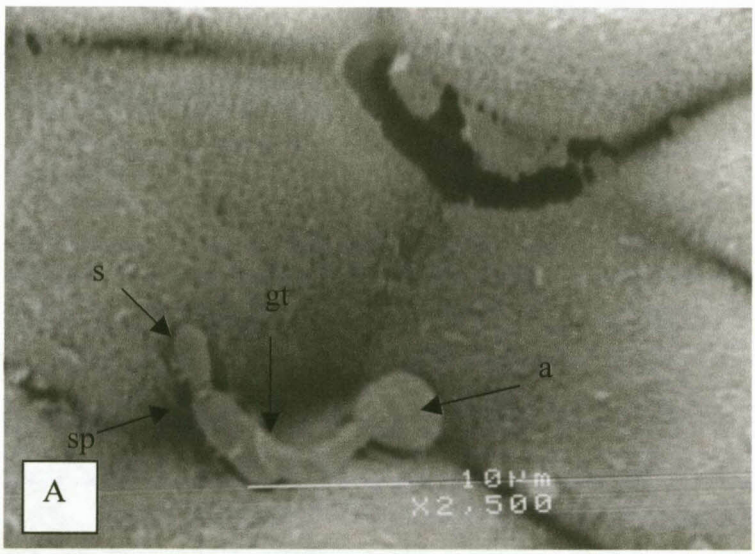

sp
s
B

**Figure 4.2** Fully formed appressoria of *C. gloeosporioides* at 24 hpi on susceptible (A) (Bar = 10μm, 2,500 x) and less susceptible (B) (Bar = 10μm, 2,500 x) lupin leaves: Appressoria (a); germtube (gt); septum (sp); and spore (s).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10μm
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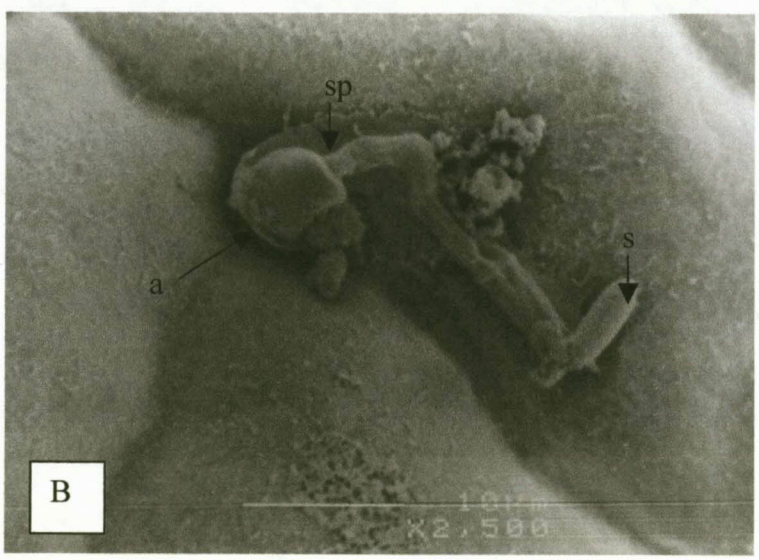
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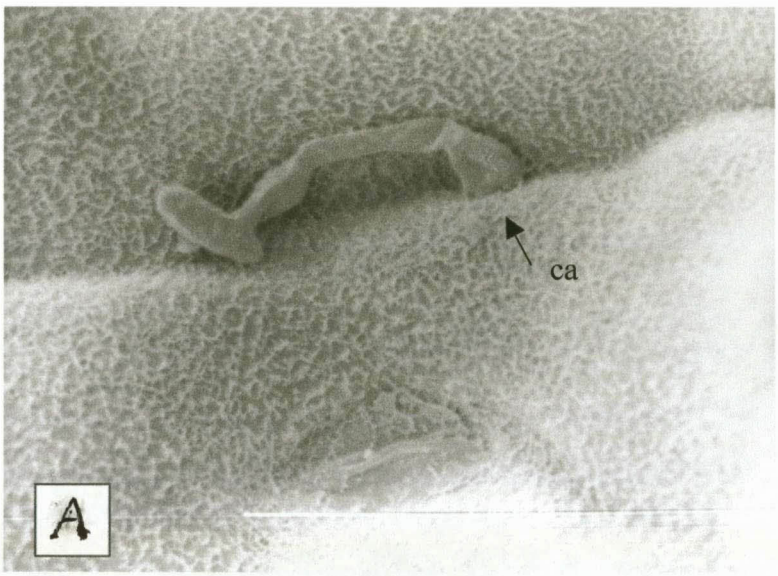
**Figure 4.3** Appressoria of *C. gloeosporioides* starting to collapse at 72 hpi on susceptible (A) (Bar = 10μm, 2,200 x) and less susceptible (B) (Bar = 10μm, 1,700 x) lupin leaves: Collapsing appressoria (ca); germtube (gt); and spore (s).


ca
A

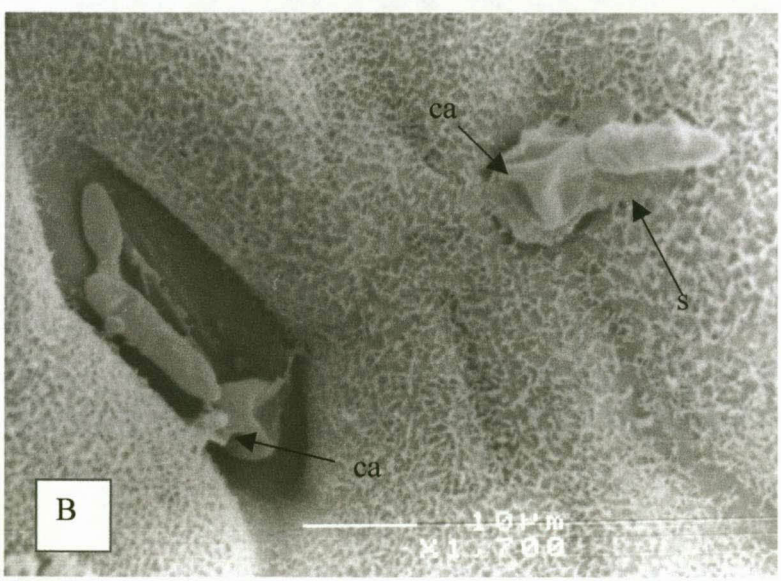

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**Figure 4.4** Development of infection peg at 48 hpi (200 x)

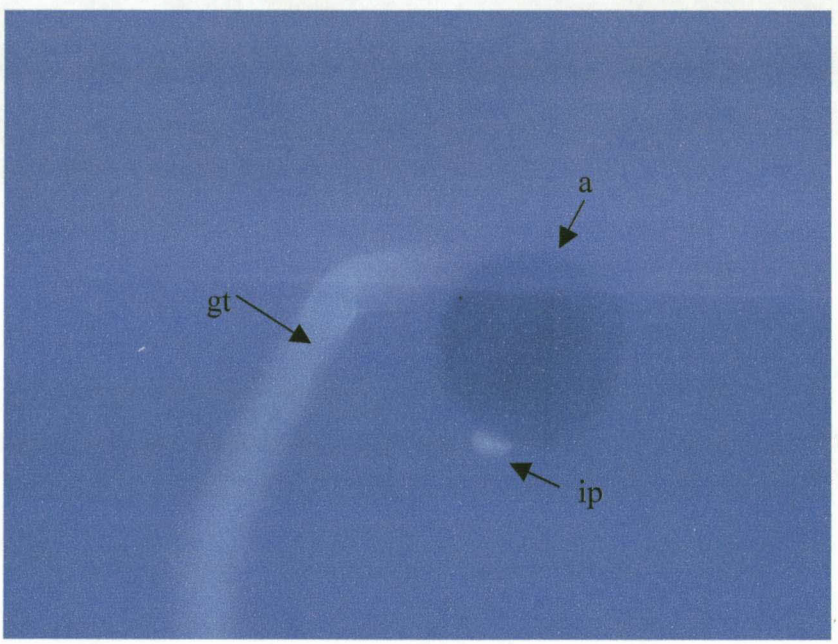
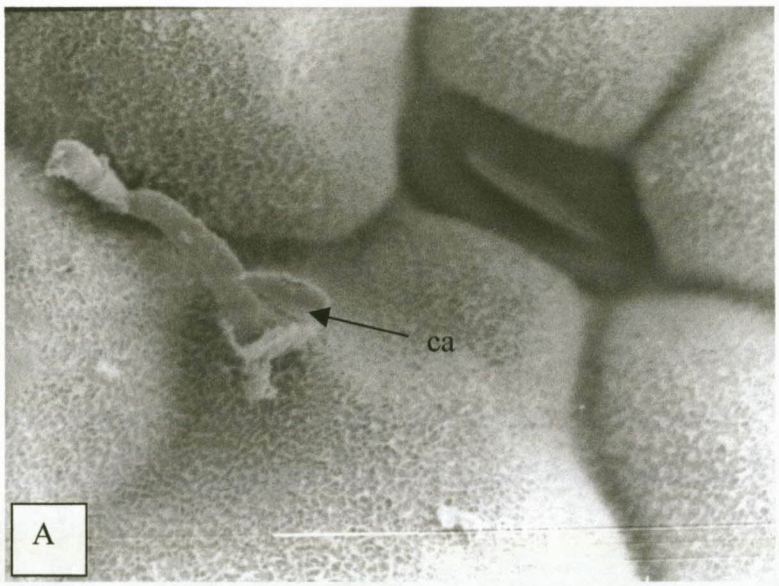

a
gt
ip

**Figure 4.5** Collapsed appressorium of *C. gloeosporioides* at 96 hpi on susceptible (A) (Bar = 10μm, 750 x) and less susceptible (B) (Bar = 10μm, 2,200 x) lupin leaves: Collapsing appressorium (ca); and septum (sp).


ca
A

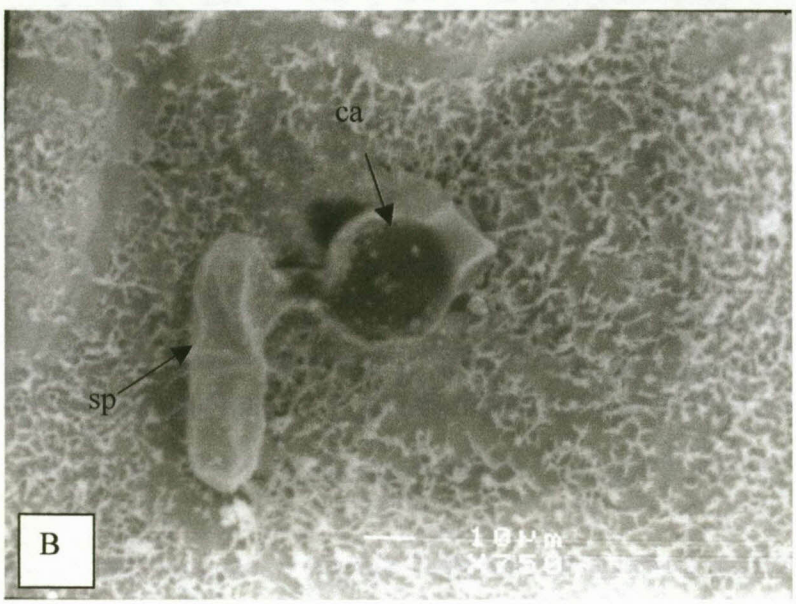
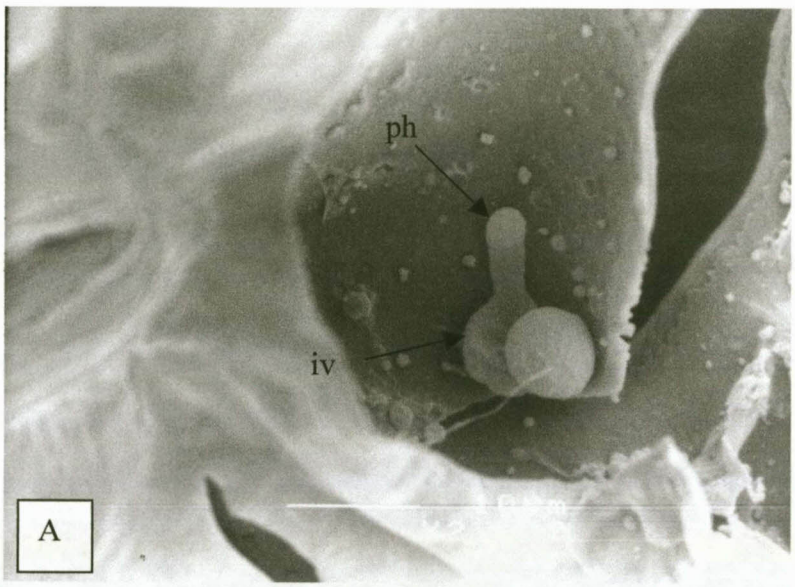
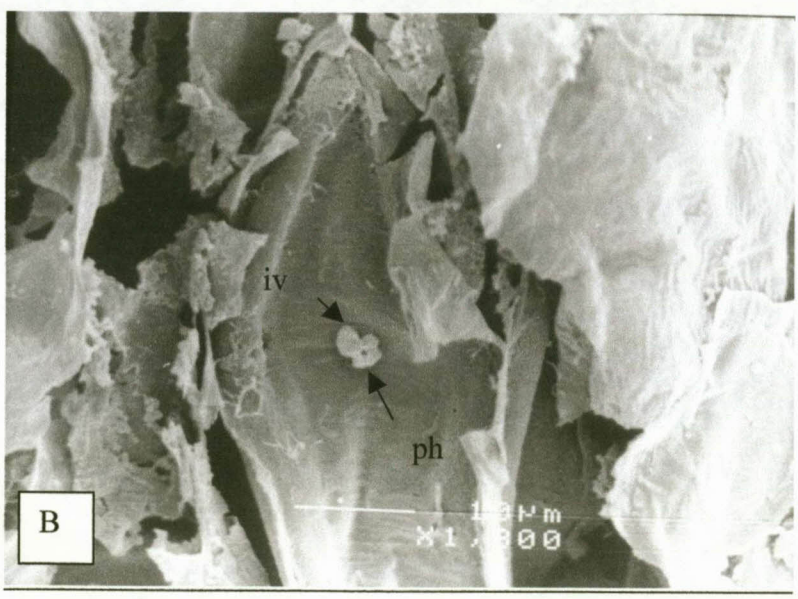

ca
sp
B

**Figure 4.6** Development of an infection vesicle and primary hypha on leaves of lupin at 96 hpi on susceptible (A) (Bar = 10μm, 2,000 x) and less susceptible (B) (Bar = 10μm, 1,800 x) lupin leaves: Sub-epidermal infection vesicle (iv); primary hypha (ph) generated from infection vesicle in an epidermal cell.


ph
iv
A


iv
ph
B

## SUMMARY

In pathogenicity studies on 14-d-old lupin seedlings, six isolates of *C. gloeosporioides* (collected in 1999 and 2000) showed no pathogenic variation on the 16 lupin cultivars tested compared to an isolate collected in 1995 at the start of the disease outbreak. *L. angustifolius* cultivars Wonga and Tanjil were consistently more resistant and *L. albus* cultvars Kiev Mutant, Swartland, CED 6150, Elsa and Vladimir were more susceptible to the seven isolates tested. This suggests that no new virulent isolates of the fungus have appeared in South Africa and that in the short term the cultivation of *L. angustifolius* cultvars, although *L. albus* cultivars is preferred, is safe from severe anthracnose attacks in this country. Continuous characterization of isolates and monitoring must however be encouraged in future in order to prevent any potential threats to the local lupin industry.

By employing the barley bioassay method of Jones & Varner (1967), gibberellin-like substances were detected in liquid culture from isolates of *C. gloeosporioides* that cause lupin anthracnose. This is also the first time that gibberellin is associated with a *Colletotrichum* spp. Its role in the pathogenesis of lupin anthracnose is unknown although some studies have established a correlation between $GA_3$ production and increased disease severity (Sunder *et al.*, 1998) and therefore might be useful for in screening of lupin cultivars for disease resistance. As gibberillins have many modes of action (Salisbury & Ross, 1992) the role of gibberellins in this specific pathogen/host interaction must be investigated. The relationship between $GA_3$ production by the pathogen and the typical twisting and bending of stems and petioles must also be determined.

At microscopic level no qualitative differences in conidial germination, appressorium formation or penetration of the epidermal cell wall by *C. gloeosprioides* in the

very susceptible *L. albus* cv Kiev Mutant and the less susceptible *L. angustifolius* cv Wonga were detected. Penetration occurred directly through both the cuticle and epidermal cell walls. In both cases small spherical vesicles with single primary hyphae were observed in the first penetrated epidermal cells. It will appear that the initial establishment of the pathogen up to vesicle formation is not related to the resistance of lupin cultivars. Post infection hyphal development up to 144 h could not be observed and might be related to an extended biotrophic relationship between the fungus and the host. Furthermore it seems like defense reactions by the plant are only activated after penetration and initial establishment of the pathogen in the host epidermis.

## OPSOMMING

In infeksiestudies op 14-d-oue saailinge van 16 lupienkultivars is geen variasie waargeneem tussen ses isolate van *C. gloeosporioides* (versamel in 1999 en 2000) en 'n isolaat versamel aan die begin van die siekte-uitbraak in 1995. Die *L. angustifolius* kultivars Wonga en Tanjil was deurgaans meer bestand en die *L. albus* kultivars Kiev Mutant, Swartland, CED 6150, Elsa en Vladimir meer vatbaar teen die sewe isolate getoets. Dit dui aan dat geen virulensieverandrings in die swam voorgekom het nie en dat alhoewel *L. albus* verkies word, *L. angustifolius* kultivars tans beskerm is teen hewige antraknose-infeksies. Voortdurende karakterisering van isolate en monitering word egter aanbeveel om die plaaslike lupienindustrie teen virulensieveranderings te waarsku.

Met behulp van die garsbiotoets van Jones & Varner (1967) is gibberelienagtige verbindings waargeneem in vloeibare kulture van *C. gloeosporioides* isolate wat lupienantraknose veroorsaak. Dit is die eerste aanmelding van 'n verwantskap tussen gibberelien en *Colletotrichum* spp. Met die uitsondering van 'n korrelasie tussen $GA_3$ produksie en verhoogde siektevoorkoms (Sunder *et al.*, 1998) is die rol daarvan in antraknose-patogenese grootliks onbekend, en kan dit moontlik van hulp wees in die evaluering van siekteweerstand. Aangesien gibbereliene baie meganismes van werking het (Salisbury & Ross, 1992) word voorgestel dat hul rol in die lupien-antraknose interaksie, met spesifieke verwysing na die verdraaiing en buiging van stamme en blaarstele, verder ondersoek word.

Op mikroskopiese vlak is geen kwalitatiewe verskille in konidiumontkieming, appressoriumvorming of penetrasie van die epidermisselwande van die vatbare *L. albus* kv. Kiev Mutuant, en meer bestande *L. angustifolius* kv. Wonga, deur *C. gloeosporioides* gevind nie. Direkte penetrasie deur beide die kutikula en epidermiswand is waargeneem. In

beide gevalle het klein, sferiese vesikels met enkel, primêre hifes in die gepenetreerde epidermisselle voorgekom. Dit het geblyk dat die aanvanklike vestiging van die patogeen nie beïnvloed is deur die weerstandsvlakke van lupienkultivars nie. Geen verder swamontwikkeling is waargeneem tot 144 h na penetrasie nie en kan dit verband hou met 'n verlengde biotrofiese verwantskap tussen die patogeen en gasheer. Dit was duidelik dat die weerstandsmeganismes eers na die aanvanklike penetrasie en vestiging van die swam in die gasheerepidermis geaktiveer word.