FUNGAL DISEASES OF PIGEONPEA IN SOUTH AFRICA

by

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PREFACE

Several members of the legume family are grown worldwide, and are second only to the cereals as a food source for humans and animals. Disease is a major constraint in the production of legumes. To reduce losses due to disease, it is important to have a sound understanding of abiotic and biotic factors contribute to disease development, and to be able determine the extent and severity of the disease. Special reference is made in this dissertation to pigeonpea (*Cajanus cajan*), a versatile legume widely cultivated in the tropics and subtropics, still considered a new crop in this country, but with production increasing in eastern and southern Africa. The purpose of these studies were to improve knowledge concerning pigeonpea, its fungal pathogens and some other factors that contribute to certain diseases of this host. The resulting knowledge could prove useful in a sustainable disease management approach for the successful cultivation of pigeonpea in South Africa. Due to the fact that each chapter in this dissertation represents an independent unit, some repetition may occur.

Chapter 1 is a review of the role-playing factors, related to the host, pathogen or environment, that contribute to disease development in legumes. These factors are in effect sub-components of the disease triangle which can be manipulated to reduce losses due to disease. Although these factors are discussed individually, their interaction is of importance.

In chapter 2, two fungal species (*Passalora cajani* and *Cercospora apii*) isolated from pigeonpea, and potential pathogens to this host, were characterized in terms of sensitivity to fungicides and physiological requirements *in vitro*. Molecular analysis was applied to corroborate the results of cultural studies.

In the third chapter, one of the newly reported diseases which occur on pigeonpea in South Africa, is investigated. Histopathology of pigeonpea rust, caused by *Uredo cajani*, was studied on six pigeonpea varieties, to contribute to better understanding of the pathogen and at the same time screening varieties for resistance.

In chapter 4 a disease rating system for the pigeonpea-rust pathosystem

was developed and used in a field trial to determine differences between varieties being tested for cultivation in South Africa. Yield and quality loss due to pigeonpea rust and the efficacy of selected fungicides was also studied.

CHAPTER 1

A REVIEW OF FACTORS INFLUENCING FUNGAL DISEASES OF PIGEONPEA AND OTHER LEGUMES

INTRODUCTION

Several members of the legume family, also referred to as Fabaceae, Leguminosae, or Papilionaceae (Michaels, 1991) are grown worldwide, throughout the tropical, subtropical and temperate regions (Ingham, 1982). Legumes are second only to the cereals as a food source for humans and animals. Legumes are characterized by the unique ability to form symbiotic relationships with bacteria in *Rhizobium* and *Bradyrhizobium* spp. which can utilize atmospheric nitrogen, enabling this family of plants to have a worldwide distribution (Koch, 1996). During this important soil-enriching process, nitrogen is fixed in root nodules (Sprent and Minchin, 1985). With nitrogen-rich plant residues remaining in the soil, the productivity of other crops grown in association with legumes, is enhanced (Koch, 1996).

In countries with poor soils, legume crops are preferred because of their soil-enriching properties (Giller and Wilson, 1991). Certain drought-tolerant legumes, possessing deep and laterally well-spread root systems, can be cultivated successfully in areas with low rainfall. Several legume types are grown to combat soil erosion, as is the case with pigeonpea (*Cajanus cajan* (L.) Millsp.) (Nene and Sheila, 1990).

Norton, Bliss and Bressani (1985) stated that legumes are a major source of calories and dietary protein in food and feed products throughout the world. Pulse legumes are planted and harvested for their seeds, both mature or immature (Nwokolo, 1996) and are especially valuable as food in areas where animal protein is scarce. Oilseed legumes contribute a major part of the world's edible oil supply (Norton *et al.*, 1985) and are used to a large extent for feeding livestock (Nwokolo, 1996). Norton *et al.* (1985) observed that apart from protein, they also supply calcium, iron, thiamine and riboflavin. Consumption of legumes is beneficial to human health, according to Sathe (1996). Apart from having the ability to lower serum cholesterol in humans, they have a high fibre content and a low sodium content. In first world countries, where other sources of protein, especially animal protein, are abundant, pea and bean cultivars are selected on the basis of their aesthetic rather than nutritional value.

Disease is a major constraint in the production of legumes (Koch, 1996) and host specificity is evident for the wide variety of pathogens that attack legumes. To reduce losses due to disease, it is important to understand factors that contribute to disease development, and to determine the extent and severity of the disease. A thorough understanding of these factors should lead to improved production of legumes.

The interaction between a host plant and an associated pathogen is influenced by factors which often determine whether or not infection occurs, the infection rate, and the changes that occur in the host as well as in the pathogen prior to and following infection. These factors are essentially related to the physiology of the host and pathogen, as well as the environment in which they interact. Although there is a high level of interaction between these respective factors, Colhoun (1973) pointed out that studies of the effects of individual factors are crucial in understanding disease incidence. Individual factors can display considerable variability which affects the degree of disease severity within an individual plant or within a plant population (Agrios, 1997).

With specific reference to host, pathogen and environment related factors, the aim of this review is to examine the many factors that need to be considered when studying diseases of pigeonpea and other legumes. Understanding the roles of the factors separately, contributes to understanding the interactions between them, and the various combinations of their respective components that give rise to different plant diseases.

HOST RELATED FACTORS

Pathogens differ with respect to the species of plants that they can attack, and which plant part they affect. Each plant species is susceptible to attack by only a relatively small number of known pathogens (Agrios, 1997). Providing a plant is genetically susceptible to a pathogen, disease incidence and severity are influenced by many host and environment related factors that can be inhibiting or conducive to disease.

Genotypic variation

Genotypic variation among species can drastically influence the natural incidence of disease. Echavez-Badel and Bosques-Vega (1998) found distinct host variation among long-day *C. cajan* genotypes in their respective levels of susceptibility to pigeonpea rust, *Uredo cajani* Syd. The early maturing variety I8-2 displayed a greater degree of *U. cajani* infection than late maturing II56.

Genetically determined host resistance is not an all-or-nothing attribute of the host (Lucas, 1998). In practice, there is a range of responses that vary from high resistance where no visible symptoms occur, to low resistance where the host succumbs to the disease. Differences in susceptibility to brown stem rot, caused by *Phialophora gregata* (Allington and Chamberl.) W. Gams (syn. *Cephalosporium gregatum* Allington & Chamberl.), exist among soybean (*Glycine max* (L.) Merr.) cultivars, but none are immune.

Inherited genetic resistance is a popular means of plant disease management because it requires low input during the growing season (Schumann, 1991). When F2 populations of nine crosses involving nine susceptible parents and one resistant parent, ICP-7065, of pigeonpea were screened in a blight nursery and selfed (Sharma, Kannaiyan and Reddy, 1982), five of the nine crosses segregated in a 1:2 pattern of true breeding resistance. Breeders and growers prefer varieties that have some disease resistance and if possible, resistance to more than one pathogen, also known as multiple-resistance (Nene, 1988).

Growth stage

Many studies have investigated the respective roles of age and senescence in the infection process and disease expression (Tschanz, 1982; Sinclair, 1991). Many hosts are susceptible to pathogens only during a certain stage of their life cycle. Host age can affect processes varying from infection and colonisation to disease expression. Sinclair (1991) has shown that *Cercospora kikuchii* (T. Matsumoto & Tomoy.) M.W. Gardner, which causes leaf spot and leaf blight in soybeans, usually induces symptoms during seedset (growth stage R3-R4), regardless of environmental conditions. The development of symptoms is associated with physiological changes in the plant during transition from vegetative

to reproductive stages. The same is true in the case of soybean rust, caused by *Phakopsora pachyrhizi* H. syd. & P. Syd., where symptom expression is delayed until after flowering (Tschanz, 1982). A delay in host maturity delays the onset of rust and also reduces the development rate. In addition, early maturing cultivars develop rust earlier and at a faster rate than late maturing cultivars (Tschanz, 1982), maturing faster to reach the physiological age that triggers onset of rust development. In contrast, soybean plants are susceptible to *Colletotrichum* spp., the cause of anthracnose, throughout the developmental stages but symptoms typically appear during the early reproductive stages on pods, stems and petioles (Manandhar and Hartman, 1999).

Latent infections of plants by pathogenic fungi are considered the highest form of parasitism (Sinclair and Cerkauskas, 1996), where the parasitic relationship eventually induces macroscopic symptoms (Verhoeff, 1974). Latent period, varying in length of time, usually ends when the plant is under stress, begins to senesce, or is killed (Sinclair, 1991). Several pathogens cause latent infections in soybeans. Sinclair (1991) found that charcoal rot, caused by *Macrophomina phaseolina* (Tassi) Goid., usually appears on soybean plants when they reach senescence or after a period of drought. Quiescent infections, however, are macroscopically visible with an arrest in mycelial development after infection, only to resume as the host reaches maturity and/or senescence (Byrde and Willets, 1977). *Phomopsis sojae* Leh. remains semi-dormant and close to the infection point of inoculation until soybean plants begin to mature, when the proliferation of the fungus in plant tissues results from multiple localized infections (Hill, Horn and Steffens, 1981).

Leaf morphology

Leaf surface. There are definite differences between adaxial and abaxial leaf surfaces which influence fungal tropisms (positive or negative primary responses of germ tubes and hyphae to a source of stimulation) (Wynn, 1981). Wynn (1976) demonstrated a correlation between lower percentage of appressorium formation with fewer stomata (30/mm^2 leaf area) occurring on the upper surface of bean *(Phaseolus vulgaris* L. 'Pinto') leaves compared to the lower surface with 130

stomata/mm^2 leaf area. Presence of trichomes also plays a role in the establishment of infection (Wynn, 1976). Low percentage of appressorium formation by *Uromyces appendiculatus* (Pers.: Pers.) Unger (syn. *Uromyces phaseoli* var. *typica* (Pers.) G. Winter.), on nonhost soybean plants was correlated with a high density of trichomes where germ tubes often grew along the trichomes and therefore did not come into contact with the leaf surface.

Leaf topography. Wynn (1976) showed that germ tube growth of *Ur. phaseoli* var. *typica* on bean leaves is related to the topography of the leaf surface. By growing at right angles to the cuticular ridges encircling the stomata, germ tubes are directed to the stomata. Directed growth maximizes the chances of locating a stoma. Anderson (1982) stated that topography of the plant/leaf surface could play an important role in disease resistance. Smooth surfaces would provide fewer locations for pathogens, free water or nutrients.

Defence mechanisms

Plants have highly efficient defence mechanisms that resist the incessant challenges by microbial organisms. Defence mechanisms discourage initial infection and may restrict growth after infection. Agrios (1997) divided defence mechanisms into two categories: (1) structural characteristics that act as physical barriers and inhibit the pathogen from gaining entrance and spreading through the plant, and (2) biochemical reactions that take place in the cells and tissues of the plant, producing substances either toxic to the pathogen or creating conditions that inhibit the growth of the pathogen in the host. Defences are also either constituent (preformed) or induced (Hammerschmidt and Nicholson, 1999).

Structural defence. Preformed defence mechanisms or constitutive resistance, associated with the plant surface, form the first line of defence against pathogens (Agrios, 1997). Pre-existing defence mechanisms include amount of wax and structure, the structure of the epidermal cell walls, size, location and shape of stomata, and the presence of thick-walled host tissues.

Attachment of fungi to the host surface is essential for penetration and utilization of the substrata (Griffin, 1994) and is a very specific process. Waxes on leaf or fruit surfaces form a water-repellent layer preventing the formation of a

water film in which fungal spores might germinate. However, binding of conidia of *Colletotrichum lindemuthianum* (Sacc. & Magnus) to bean hypocotyls increases on tissues covered with wax. This hydrophobic bonding is specific in most cases. Cook (1980) associated resistance to peanut rust, caused by *Puccinia arachidis* Speg., with increased water repellency as leaves aged, causing run-off to remove an increasing proportion of the inoculum, reducing appressorium formation. Trichomes may also have a water-repelling effect, reducing infection by fungi that require a film of water in which to germinate (Agrios, 1997). Leaves, fruit, flowers and stems are covered by a cuticle, containing the structural component cutin, an insoluble polymer, embedded with a complex mixture of hydrophobic materials collectively called wax (Kolattukudy and Köller, 1983; Mauseth, 1991). A thick cuticle may increase resistance but not prevent infection. Campbell, Huang and Payne (1980) claimed antifungal activity in a variety of molecules present in the cuticular layers of plants, which might be a preformed resistance mechanism (Anderson, 1982) that possibly limits enzymatic degradation of cell wall polysaccharides. Thick, tough epidermal walls make direct penetration by pathogens difficult or impossible and act as cellular defence structures.

When fungi attempt to penetrate, or actually penetrate epidermal cell walls, a distinct “halo” can be observed around the penetration point (Heath, 1980). Silicon deposition has been reported on and within the walls of French bean (*P. vulgaris*) mesophyll cells as a response to infection by *Ur. phaseoli* var. *vignae* (Heath, 1979 in Heath, 1980). The French bean does not normally have silicified walls. The susceptibility of bean (*P. vulgaris*, ‘Red Kidney’) seedling hypocotyls to *Rhizoctonia solani* Kühn decreases, as calcium content in the hypocotyls increase with age, until they are about three weeks old (Bateman and Lumsden, 1965). This increase in calcium is accompanied by thickening of the cuticle of the hypocotyls, which in turn decreases the quantity of host exudates (Stockwell and Hanchey, 1982) necessary for formation of infection cushions by *R. solani* (Reddy, 1980 in Stockwell and Hanchey, 1984). Bateman and Lumsden (1965) showed that pectolytic enzymes produced by *R. solani* are unable to macerate calcium pectate in older bean hypocotyls.

Physical barriers exist in plants and when cell walls mature, lignification may take place (Esau, 1977). The smaller lesions caused by *Co. lindemuthianum* on

older bean hypocotyls result from an apparent inability of the mycelium to penetrate lignified fibres and xylem elements (Griffey and Leach, 1965 in Ride, 1983) that form as the bean plant ages. Many pathogens simply enter through the stomata and the structure of the stomata may confer resistance to pathogen attack. The lack of response from *Ur. phaseoli* germ tubes to stomata of wheat is attributed to differences in stomatal structure (Wynn, 1976). The stomata of bean leaves are surrounded by prominent stomatal lips on the guard cells, which induce appressorium formation, while those of wheat leaves have inconspicuous lips which are concealed at the inner edges of the guard cells, and the pathogen shows no response (Wynn, 1976).

Preformed defence mechanisms can be induced by penetration of the pathogen and the various degrees of infection it causes (Agrios, 1997). Plants respond to invasion by forming histological defence structures. One of the most common changes in a nonhost or resistant plant after inoculation is the formation of a papilla, an apparent thickening of the plant cell wall due to depositing of material between the cell wall and the plasmalemma (Heath, 1980). This deposition of lignified material may continue until the pathogen is encased. Heath (1974) reported a collar of papilla material around the haustorial neck of the rust fungus, *Ur. phaseoli* var. *vignae* when the pathogen grew through the preformed papilla.

Cytoplasmic defence reactions involve the cytoplasm of cells under attack (Agrios, 1997). Where necrosis occurs, ultrastructural changes are seen in the adjacent, seemingly healthy cells (Heath, 1980), presumably in response to products liberated during cell death. Mercer *et al.* (1974) in Heath (1980) found that in resistant French bean infected with *Co. lindemuthianum*, cells next to necrotic ones had an increased volume of cytoplasm, and contained unusual convoluted nuclei. The death of invaded cells may protect the plant from further invasion, and is referred to as a hypersensitive reaction (HR).

Biochemical defence. Resistance of a plant to a pathogen not only depends on structural barriers but also on biochemical substances produced inside cells before and after infection (Agrios, 1997). Heath (1977) postulated that the observed lack of haustorium formation by rust might in some instances be due to the absence of

specific receptors responsible for the stimulation of haustorial mother cells to form haustoria. Such receptors are essential for pathogenesis and may prevent the establishment of a parasitic relationship (Schlösser, 1980). Most fungi secrete an array of hydrolytic enzymes that often diffuse into host tissue in advance of the pathogen and are necessary to secure a nutritional base. The inhibition of these enzymes by the potential plant host, can be a defence mechanism that restricts pathogenesis.

Metabolic defences induced by pathogens include changes in host physiology and the production of phytoalexins, phenolic compounds and host proteins. The activation of host defence mechanisms often have an effect on the allocation of resources within the host. Respiration necessary for plant growth and maintenance, constitutes a major drain of available carbohydrate resources (McLaughlin and Shriner, 1980). Increased respiration following injury is a well-recognized plant response and when injury is disease induced, it may reflect increased metabolic activity by the host, the pathogen, or both, that requires the increased allocation of energy to the infection site.

Plant defence mechanisms can involve the localised production of phytoalexins, which causes necrosis to cells adjacent to the infection site (Carroll, 1991). By definition, phytoalexins are secondary metabolites, compounds which are not obviously essential to normal growth and metabolism of the producing organism (Paxton, 1980; Martin and Demain, 1980 in Stoessl, 1982), synthesized by and accumulating in plants after exposure to microorganisms. Phytoalexin production may be induced by fungal components, called elicitors (Keen, 1975 in Yoshikawa, 1983). Ayres *et al.* (1976) in Yoshikawa (1983) extracted elicitors of glyceollin, a soybean phytoalexin, from the cell walls of *Phytophthora* Drechs. *megasperma* f. sp. *glycinea* T. Kuan & DC Erwin. Evidence suggests that the elicitor activity resides in the glucan component of the cell walls. The soybean plant recognizes and responds to compounds (elicitors) produced by *Ph. megasperma* var. *sojae* by the accumulation of glyceollin (Frank and Paxton, 1971 in Paxton,1983). Anderson (1980) in Yoshikawa (1983) found that glucans from *Co. lindemuthianum* elicited phaseollin production and hypersensitive tissue browning in green bean. A notable feature of the subfamily Papilionoidae is the widespread ability of its species to produce isoflavonoids, a group of compounds

which occur sporadically elsewhere in the plant kingdom (Ingham,1982) and that has been associated with plant defence systems (Armero *et al.*, 2001) .

Phytoalexin production can lead to a hypersensitive reaction in the host plant. Phytoalexin production by French bean has been studied extensively. Most studies comparing the mechanisms of resistance and susceptibility of *P. vulgaris* to *Co. lindemuthianum*, the cause of anthracnose, has been done on the hypocotyls. *Co. lindemuthianum* spores germinate within 48 h after inoculation to produce similar numbers of appressoria on resistant and susceptible plants (Mansfield, 1982). Only when infection hyphae penetrate underlying epidermal cells do differences in cultivars become evident. In susceptible cultivars, developing intracellular hyphae cause no observable host response and continue to grow biotrophically for several days. Only after extensive colonisation does the tissue collapse, infected cells die, and lesions appear. In resistant cultivars the initially infected cell, together with perhaps one or two adjacent cells, die and turn brown soon after infection, restricting growth to the infection site (Elliston, Kuć and Williams, 1971), a process referred to as hypersensitivity. Early studies showed that the accumulation of phaseollin, the main phytoalexin produced by French bean hypocotyls (Bailey, 1974 in Mansfield, 1982), was associated with cell death and browning in hypocotyls of both resistant and susceptible plants. It was not present during the biotrophic phase but only accumulated after the death of infected tissues (Bailey *et al.*, 1980 in Mansfield, 1982) and inhibition of fungal growth started shortly after phytoalexin accumulation.

Phenolic compounds normally found in plants are thought to function as preformed inhibitors associated with non-host resistance (Bailey and Mansfield, 1982). Others are formed in response to the ingress of pathogens and they are considered to be part of active defence mechanisms. Differentiation of the responses of plants to pathogens based on host and nonhost interactions was argued by Heath (1980). In such relationships the responses are characterized by early accumulation of phenolic compounds at the infection site and limited development of the pathogen due to rapid cell death (Fernandez and Heath, 1988). In a study on the defence mechanisms of chickpea (*Cicer arietinum* L.) against *Ascochyta rabiei* (Passerini) Labrousse, Sindhu *et al.* (1998) found that after inoculation, total phenols increased more in resistant than in susceptible cultivars.

Similar patterns shown by peroxidase and beta-1,3-glucanase, indicated that these enzymes play a role in defence against the pathogen via phytoalexin or phenolic biosynthetic pathways.

Plant hosts produce defence proteins during attack from pathogen enzymes. Hydroxyproline-rich glycoproteins (HRGPs) are present in low amounts in the cell walls of higher plants (Esquerré-Tugayé *et al.*, 1992). O'Connell *et al.* (1990) in Esquerré-Tugayé *et al.* (1992) showed HRGPs to be localized at sites where *Co. lindemuthianum* is restricted, for example in papillae and in bean cell walls undergoing a hypersensitive response. It is speculated that the HRGPs increase the structural resistance of cell walls but the precise mode of action is unknown. Host plants also secrete defence proteins at the host-pathogen interface (Esquerré-Tugayé *et al.*, 1992), which include plant hydrolases and protein inhibitors of fungal hydrolases. Two endo β-1,3-glucanases are induced during the early stages of the hypersensitive response of bean against *Co. lindemuthianum* (Daugrois *et al.*, 1992). These enzymes are thought to exert their hydrolytic activities on fungal cell walls.

Resistance

Many forms and levels of resistance operate in host plants and resistance to a pathogen is the ultimate tool in disease management.

Induced resistance. Any of the above-mentioned induced defence mechanisms can be seen as induced resistance. Induced resistance in *P. vulgaris* 'Perry Marrow' to bean anthracnose, was recently demonstrated by Rahe *et al.* (1969) in Elliston *et al.* (1971). Elliston *et al.* (1971) showed that the fungus (a non-pathogenic race) stimulates the plant cells to respond hypersensitively. The penetrated cells granulate and turn brown and contain the fungus in induced resistance interactions, thus when the protected cells are inoculated with the pathogenic race of the fungus, infection but not proliferation of the pathogenic race occurs. In another example of resistance, the collapse of cells adjacent to those containing haustoria is a host resistance mechanism. Haustoria that result from the direct penetration of leaf surfaces by *Ce. kikuchii*, are restricted in their subsequent growth in soybean foliage by such cell collapse (Orth and Schuh,

1992).

Genotypic resistance. Often real farm situations require cultivars having combined resistance to more than one disease, this being known as multiple-disease resistance (Nene, 1988). Because the range of pathogens that attack legumes is so wide, multiple-disease resistance is much needed in resource-poor countries. For example some cultivars of chickpea are resistant to powdery mildew and show only traces of rust, while others are severely affected by both diseases (Nene, 1988).

Many legume cultivars are resistant to diseases and the search for resistance genes continues. Lentil (*Lens culinaris* Medik.) cultivars that are resistant to rust and Ascochyta blight, have been released in several countries and resistant sources to vascular wilt are being exploited (Erskine *et al.*, 1994). The primary means of controlling the two most important crop damaging diseases on pigeonpea, fusarium wilt (*Fusarium udum* E.J. Butler) and Cercospora leaf spot (*Cercospora cajani* Hennings), is to plant resistant varieties (Agricultural News, 2000).

Host exudations

The chemical environment of the host plant surface can increase or decrease the rate of germination of fungal propagules and formation of infection structures (Agrios, 1997). Dodman (1978) showed that host exudates can either inhibit or stimulate germination. Large amounts of readily usable sources of carbon and nitrogen become available for the pathogen. Rovira (1965), as cited by Dodman (1978), stated that exudates from seeds and roots can stimulate spore germination. Chlamydospores of *Fusarium solani* (Mart.) Sacc. f. sp. *phaseoli* (Burkholder) germinate close to bean seeds and the tips of primary roots, but not around older roots or further away in soil (Schroth and Snyder, 1961 in Dodman, 1978). Exudates from seeds were subsequently shown to contain sugars and amino acids required for germination (Schroth *et al*., 1963 in Dodman, 1978). The presence of higher levels of sugars and amino acids in exudates of a susceptible chickpea (*Ci. arietinum*) cultivar, stimulated both chlamydospore germination and germ tube growth of *Fusarium oxysporum* Schlechtend.: Fr. f. sp. *ciceris* (Padwick)

Matuo & K. Sato compared with that from a resistant cultivar (Mahakul *et al.*, 1996).

According to Ruan, Kotraiah and Straney (1995) pisatin, a defence-related isoflavonoid phytoalexin produced by garden pea, stimulates spore germination of *Fusarium solani* Schlecht. f. sp. *pisi* (van Hall) W.C. Snyder & H.N. Hans, pathogenic to peas (*Pisum sativum* L.*)*. They concluded that flavonoids in legume root exudates may be perceived as a signal in plant-microbe interactions, for initiating pathogenic fungal interactions, with the fungus having the ability to tolerate phytoalexin inhibitory action.

In addition to plant exudates inducing germination, there are also some that are inhibiting to germination. Kraft (1977) has shown that in Fusarium root rot of peas, the pigment delphinidin is produced in the testa of certain types of peas which inhibits the germination of conidia. Hafiz (1952), in Weinhold and Hancock (1980), indicated that malic acid, secreted by chickpea leaves, can inhibit spore germination of *Mycosphaerella rabiei* Kovachevski. Schneider and Sinclair (1975) found that young cowpea (*Vigna unguiculata* L. Walp.) leaves, which are resistant to Cercospora leaf spot, possess toxic substances in leaf diffusates that can inhibit conidial germination of *Cercospora canescens* Ellis & Martin.

Nutrients

According to Huber (1980), plant nutrition determines resistance or susceptibility to disease by influencing histological and morphological structures or properties, the function of tissues to hasten or slow pathogenesis, and the ability to survive attack from the pathogen. Non-availability of nutrients can have two outcomes: firstly, the host cannot synthesize barriers and thus remains susceptible. Secondly, resistance may result from the absence of nutrients that are necessary for pathogenic activity. Mineral elements are directly involved in all mechanisms of defence as integral components of substrates, cells, enzymes, electron carriers, or as activators, inhibitors, and regulators of metabolism.

All grain legumes classified taxonomically within the Leguminosae, subfamily Papilionoideae, form symbioses with *Rhizobium* and *Bradyrhizobium* spp. and are involved in nitrogen fixation (Sprent and Minchin, 1985). Mineral

nutrient deficiencies are major constraints, limiting nitrogen fixation in legumes (O'Hara, Boonkerd and Dilworth, 1988). Nitrogen promotes vigorous growth, delays maturity, is essential for the production of amino acids, phytoalexins, and phenols (Huber, 1980) and also plays an essential role in nitrogen fixation (Giller and Wilson, 1991). High levels of phosphorous in soil induce calcium deficiency that has been shown to reduce nodulation of legumes by *Rhizobium* (Huber, 1980). Magnesium and potassium decrease the calcium content of peanut pods, predisposing them to pod breakdown by *Rhizoctonia* and *Pythium* spp.

The general vigour and phenological development stage of plants influence their capacity to defend themselves (Huber, 1980). A nutrient-stressed plant is more vulnerable to disease than one at a nutritional optimum, yet some mineral elements in excess can predispose plants to disease. Correct nutritional balance can therefore lead to enhanced physiological resistance due to the production of pathogen inhibitors.

Mineral nutrients may render certain substrates less accessible to a pathogen. Calcium, and sometimes magnesium, reduce susceptibility of castor bean (*Ricinus communis* L.) to *Botrytis cinerea* Persoon : Fries and bean to *R. solani* by protecting pectic materials from maceration by extracellular enzymes (Huber, 1980). The presence of certain mineral elements can also result in poor survival of pathogens. Sadasivan (1965) reported that the control of Fusarium wilt of red-gram (pigeonpea) with manganese is associated with restricted saprophytic survival of *F. udum*. Saprophytic activity of *F. udum* is also reduced by boron and zinc.

Plant physiology

Physiological and physical changes in plants trigger changes in the equilibrium between host, pathogen and environment. Plant productivity and growth can be altered by phytopathogens, that alter cellular processes of the host (Hutcheson and Buchanan, 1983). Mignucci and Boyer (1979), in Hutcheson and Buchanan (1983), reported that fungal infection induces a change in the photosynthetic rate of intact leaves. In most cases there is a reduction in the rate of photosynthesis. Reduction in photosynthesis efficiency may be due to diversion

of resources during infection (McLaughlin and Shriner, 1980) and different pathogens cause different patterns of impact on photosynthetic competence which relates to pathogenic events associated with the mode of nutrition for each pathogen (Lopes and Berger, 2001). *Colletotrichum lindemuthianum* affects photosynthesis most during its necrotic phase of infection (Bailey *et al.*, 1992). Bassanezi *et al.* (1997), in Lopes and Berger (2001), reported a reduction in photosynthetic and transpiration rates for *P. vulgaris* plants with different levels of anthracnose severity, as well as for plants with rust infection, caused by *Ur. appendiculatus*. Both rust and anthracnose induced increased rates of dark respiration. Livne and Daly (1966), in McLaughlin and Shriner (1980), examined the translocation of photosynthates in bean (*P. vulgaris*) and found that rust-infected leaves acted as sinks drawing photosynthate away from adjoining healthy foliage, at the expense of young, actively growing leaves. Ascochyta blight caused by *Mycosphaerella pinodes* (Berk. & A. Bloxam) Vestergr. alters carbohydrate metabolism, protein remobilisation and free amino acid translocation from hulls and seeds of dried-pea (Garry *et al.*, 1996) and results in a reduced carbohydrate and nitrogen content of seeds.

PATHOGEN RELATED FACTORS

Most plant diseases are caused by fungi and more than 8000 of the nearly 70,000 described species of fungi are known plant pathogens (Sinclair and Hartman, 1999). Disease is often the major grain yield reducing factor for legumes in many growing areas (Porta-Puglia and Aragona, 1997). Legumes are subject to soilborne diseases, including seed and seedling blights (*Pythium* and *Rhizoctonia* spp.), root rots (*Fusarium, Pythium, Macrophomina* and *Phoma* spp.) and wilts (various *formae speciales* of *F. oxysporum*), as well as foliar diseases. Foliar diseases include powdery and downy mildew, blight, grey mould, rust and anthracnose.

Inoculum concentration

At higher inoculum concentrations germination inhibitors or competition for infection sites can reduce fungal infection. Singh *et al.* (2000) found germ tube development of *Alternaria tenuissima* (Kunze) Wiltshire on pigeonpea to be faster at lower spore concentrations, the length of germ tubes were longer and germination increased. Cook (1980) found that *Pu. arachidis* spores in clumps and dense patches on inoculated peanut (*Arachis hypogaea* L.) leaves failed to germinate due to the presence of the self-inhibitor, methyl *cis*-3, 4-dimethoxycinnamate, in spores and the surrounding water.

Spore dormancy and germination

The germination of spores is the transition from dormancy to active growth and crucial for propagation of fungal pathogens (Kolattukudy and Köller, 1983). Fungal spores do not grow actively and simply serve for dispersal and/ or survival in extreme environmental conditions (Griffin, 1994). Spore dormancy may be imposed by exogenous nutritional conditions or by endogenous control. The widespread phenomenon of soil fungistasis may inhibit fungal spores and is in turn affected by soil pH, moisture content of soil and availability of some nutrients. Plant surfaces may also contain fungistatic or fungitoxic substances (Kolattukudy and Köller, 1983).

Many plant pathogenic fungi remain dormant until a host plant is encountered. Two sources of inoculum for *Co. lindemuthianum* are conidia, produced in acervuli, and ascospores, produced in perithecia. Conidia and ascospores in young fruiting bodies are encased in a hydrophilic mucilaginous material, also referred to as a spore matrix (Louis, Chew and Lim, 1988 in Bailey *et al.*, 1992). The matrix has several roles, including inhibition of premature spore germination, thus ensuring distribution of inoculum (Louis and Cook, 1985 in Bailey *et al.*, 1992). Blakeman (1980) found that early events in spore germination include the mobilization of internal nutrients but that additional exogenous nutrients are also required.

Colonisation

Colonisation by a fungal pathogen can be limited to one cell or one cell layer, depending on the pathogenic relationship that is established between the host and the pathogen. This can include the formation of intracellular, intercellular hyphae and/or specialized infection structures such as haustoria at host-pathogen interfaces. Many economically important plant pathogens establish a biotrophic/ parasitic association with their host, where infected cells and tissues remain alive and active (Manners and Gay, 1983). This entails a precise and intimate relationship of the two organisms that seems to suggest that the extent of colonisation is determined by strategies of the pathogen.

Intracellular hyphae. *Colletotrichum lindemuthianum* on *P. vulgaris* exhibits a two-phase infection process (Bailey *et al.*, 1992). The initial phase involves intracellular, biotrophic growth that is symptomless (O'Connell, Bailey and Richmond, 1985), the fungi apparently obtaining nutrients from the apoplast without activating host responses to inhibit pathogen growth (Bailey, 1983 in Bailey *et al.*, 1992).

Intercellular hyphae. When compared to the hyphae of saprophytes, intercellular hyphae of pathogens do not possess any features that clearly indicate involvement in nutrient transfer (Manners and Gay, 1983). *Colletotrichum lindemuthianum* has two infection phases (Bailey *et al.*, 1992); firstly, having established biotrophic infection, the pathogen is transformed into an aggressive pathogen that grows both intracellularly and intercellularly, causing extensive cell death (O'Connell *et al.*, 1985). *Colletotrichum lindemuthianum* then acts as a necrotrophic pathogen and visible disease symptoms are produced.

Haustoria. Bushnell and Gay (1978) defined a haustorium as a specialised organ, formed inside a living host cell as a branch of an extracellular (or intercellular) hypha or thallus, which terminates in the host and plays a role in interchange of substances between the host and the fungus. Haustoria enter individual cells without breaching the host plasmalemma but occupy an invagination developed by its proliferation (Manners and Gay, 1983). In cells infected by powdery mildew and rust haustoria, the plasmalemma adjacent to the haustorium is highly differentiated, while the rest remains normal, as is the case with powdery mildew of pea, caused

by *Erysiphe pisi* DC. (Gil and Gay, 1977 in Manners and Gay, 1983). The haustoria of this pathogen are commonly in the mesophyll, where the host cells are engaged in photosynthesis and have direct connections with each other (Manners and Gay, 1983). Manners and Gay (1978), as cited by Manners and Gay (1983), demonstrated nutrient absorption by haustoria of the powdery mildew fungus, *E. pisi* by extracting haustorial components from infected photosynthesizing leaves. Similarly, labelled sucrose of host origin, was detected in the mycelium of the *E. pisi* (Manners and Gay, 1982 in Manners and Gay, 1983).

Enzymes

Pathogens depend on the secretion of extracellular enzymes that hydrolyse polymers and allow access for invading the cells and the release of substrates for pathogen metabolism (Boyer, 1995). Pathogens can produce extracellular enzymes to damage the host (Huber, 1980) and enable them to penetrate cell walls of their hosts (Anderson, 1978; Cooper, 1984). This, together with the use of toxins, is one of the most important mechanisms that pathogens use to attack the host. Most pathogens need certain enzymes to infect the host without causing too much damage. Pathogens are mostly kept at bay by the pectin in the cell walls and removing pectin allows larger molecules to penetrate (Baron-Epel, Gharyal and Schindler, 1988). Evidence suggests that changes in host cell walls during maturation increase their ability to elicit the production of wall-degrading enzymes by a pathogen. Various pectin-degrading enzymes are among the first enzymes to be introduced by invading pathogens. One of the predominant polysaccharides of the plant cell wall is pectin, a methylated heteropolymer containing a α-1,4-linked galacturonic acid (Carpita and Gibeaut, 1993 in Dumas *et al.*, 2000). Since this material consists of a mechanical barrier as well as a carbon source, many phytopathogenic microorganisms secrete an array of enzymes, including pectin lyase and polygalacturonase. These enzymes play a dual role in pathogenicity by inducing cell wall degradation (Collmer and Keen, 1986) and the expression of defence genes in the host plant (Hahn, Darvill and Albersheim, 1981). Growth through plant cell walls can appear largely mechanical during penetration of bean by *Co. lindemuthianum*. Hyphae constrict markedly (Cooper, 1983) but

endopolygalacturonase (endoPG), which degrades pectic polysaccharides and causes cell death, is one of the first enzymes secreted by the latter pathogen (Anderson, 1978; English *et al.*,1972 in Esquerré-Tugayé *et al.*, 1992). Plant pathogens produce an array of enzymes capable of attacking the host's plant cell components, only pectic enzymes however have a convincing role in pathogenesis (Collmer and Keen, 1986). Anderson (1978) observed the rapid induction of pectic enzymes with the bean pathogen *Co. lindemuthianum,* grown on bean cell wall, although these enzymes where also induced when grown on corn, thus being non-specific.

Phytoalexins are toxic to most fungi, however pathogens have the ability to detoxify phytoalexins formed by its specific host (VanEtten, Matthews and Smith, 1982; VanEtten, Matthews and Matthews, 1989). *Fusarium solani* f. sp. *phaseoli* has the ability to detoxify French bean isoflavonoid kievitone to kievitone hydrate (VanEtten *et al.*, 1982) and phaseollidin (Turbeck, Smith and Schardl, 1992) by extracellular enzymes (hydratases) secreted into infected tissues. Most pea pathogens were found to be able to detoxify pisatin, an isoflavonoid phytoalexin produced by garden pea (*Pi. sativum*), by demethylation that results in the less toxic compound 3,6a-dihydroxy-8,9-methylenedioxypterocarpan (Delserone *et al.*, 1999).

Toxic metabolites

Scientists have been intrigued by the mechanism used by pathogens to invade plant tissue since the early part of this century (Durbin, 1983). One of the important disease causation mechanisms is the concept that pathogens can produce substances toxic to the host. These toxins are injurious to the host and can reproduce commonly observed symptoms of disease but are not always responsible for them. They can also induce ultrastructural abnormalities which may or may not lead to visual effects (Hanchey, 1981 in Durbin, 1983). Most species of *Fusarium* produce toxic metabolites (Claydon and Grove, 1984 in Carroll, 1991). Trichothecenes are one of the important classes of fungal toxins and are among the most potent inhibitors of protein synthesis known (Jarvis *et al.*, 1987 in Carroll, 1991). This activity contributes to their notoriety as mycotoxins

and they have allegedly been used as agents in biological warfare. Nutsugah *et al.* (1994) extracted a toxin from spore germination fluids of *A. tenuissima* which selectively induced necrosis on pigeonpea leaves in a detached leaf assay. The pathogen produced toxins on detached leaves of the cultivar Bahar at a low concentration. On the resistant line Tanzania and nonhosts a toxin concentration of at least 20 000x higher was tolerated by the hosts.

Nutrients

Nutritional state of the host plant is decisive for the parasitic success of many pathogens (Huber, 1980). Nutrients may act directly on the germination, growth and penetration of a pathogen. Exogenous nitrogen and carbon are required for germination of chlamydospores of *F. solani* f. sp *phaseoli* and favour early penetration and pathogenesis (Huber, 1980). These external sources of nutrients may be present in the soil or from host exudates. Mineral elements may inhibit or activate the extracellular enzymes that pathogens use to damage the host with. Reduced Fusarium wilt, associated with minor elements such as zinc and iron, has been attributed to reduced virulence through enzyme inhibition and reduced synthesis of pectolytic enzymes (Huber, 1980).

ENVIRONMENT RELATED FACTORS

Environmental stresses may influence plant disease through the effect on host susceptibility, effect on the pathogen, or the effect on the host-pathogen interaction (Yarwood and Hooker, 1966 in Schoeneweiss, 1975). Although predisposition implies an effect on the host rather than on the pathogen, effects of biological stresses on host-pathogen interaction are often difficult to separate from effects on the host only.

Temperature

Temperature has been regarded as one of the most important variables affecting the development of biological systems (Colhoun, 1979). It has a definite

effect on occurrence and development of diseases (Colhoun, 1973), by affecting the pathogen, the host or the host-pathogen interaction. Temperature stress can be caused by low temperatures or high temperatures. Plants, predisposed by heat stress, causing phytoalexin reduction or suppression in beans, and increase in susceptibility to rust, regained normal resistance within three to five days after exposure to heat stress (Chamberlain and Gerderman, 1966 in Schoeneweiss, 1975). High temperature stress can be seen as a less important factor since plants are usually able to cool themselves down by respiration.

Butler and Jadhav (1991) observed that rust severity on groundnut plants inoculated with *Pu. arachidis,* was greatest between 17 and 25 ˚C, whereas few lesions developed around 10 and 30 ˚C. Understanding how temperature influences latent period is fundamental to improving disease control (Wadia and Butler, 1994). For rust, early leaf spot and late leaf spot in groundnuts, the longest latent periods occurred at the lowest temperatures and the shortest periods between 20 and 30 ˚C for all three diseases. High temperatures also brought about a noticeable reduction in development rate for rust. Abawi and Grogan (1975) showed temperature to exert a significant effect on apothecial formation, ascospore germination and growth by *Sclerotinia sclerotiorum* (Lib.) De Bary (*Whetzelinia sclerotiorum* (Lib.)), and initiation of infection and expansion of lesions on beans. Apothecia formation from sclerotia was highest at 11 ˚C, with no growth at either 5 or 30 ˚C. When transferred from 30 ˚C to 11 ˚C, a low number of apothecia was formed. Similarly, ascospore growth is temperature dependant, with an optimum differential germination rate of 98 % at 25 ˚C at 6 h post-inoculation, whereas only 6 % at 30 ˚C.

Tschanz (1982) found that temperatures favourable to the growth and development of soybean plants also favour rust epidemics. Low temperatures (< 15 ˚C) greatly reduced the rate of lesion number increase or prevented lesion development, however, these temperatures also severely affect soybean growth and development.

Nutrients

Lewis (1953), in Schoeneweiss (1975), proposed a balanced hypothesis of parasitism. Host-parasite relations are governed by a combination of the biochemistry of the host and the nutritional requirements of the parasite. Nutrients present in metabolic concentrations in the host sometimes cause inhibition of parasites and so a certain nutrient imbalance may be a necessary pre-requisite for infection. It is clear that a nutrient deficiency as well as nutrient imbalance in the host, can have serious effects on the host physiology which in turn can influence the host-parasite interaction.

Amending soils, infested with *F. udum*, with boron, manganese and zinc, decreased the percentage of pre-emergence wilt in *C. cajan* as compared with the control (Sadasivan, 1965). These elements, especially zinc, hastened the disappearance of the pathogen. Sindhan and Parashar (1989) studied the effect of macro- and micronutrients on the development of powdery mildew of pea. *Erysiphe pisi* caused most disease in plants supplied with high N and Fe, with low doses of P, K, Zn and Cu. The opposite was true for low N and Fe, coupled with double doses of P, K and Zn. In the latter case, disease intensity was significantly reduced. In contrast, cowpea seedling rot, caused by *R. solani*, was increased by various N, P and K combinations, except N+K (Walia, Sunder and Grover, 1992), and increased at low rates of micronutrient applications. Infection decreased with high rates of micronutrient application.

Free water/Moisture

Relative humidity plays a role in the presence of water on plant surfaces which directly influence infection. After inoculation of groundnut with *Pu. arachidis*, Butler and Jadhav (1991) found that a minimum period of leaf wetness was necessary for infection. Cook (1980) showed that not all germ tubes arising from germinating *Pu. arachidis* spores formed appressoria. The probability of appressorium formation is less likely on leaf surfaces which are water repellent. Not only is atmospheric humidity associated with sporulation of pathogens but inoculum dispersal of many species is achieved by splashing raindrops or water droplets (Colhoun, 1973). Moisture is a limiting factor in the development of white

mould caused by *S. sclerotiorum* on beans, as infection can only occur if free moisture is maintained for a relatively long period at the interface of host tissue and inoculum (Abawi and Grogan, 1975). Even after lesion formation, development would stop abruptly as inoculated tissue became dry. Even relative humidity near to 100 % was not sufficient for lesion initiation and development. Arrested lesions or dry colonized tissues required 48 to 72 h of continuous moisture before lesion initiation or expansion was evident.

Host plants are often predisposed to disease during water deficiencies in the soil (Boyer, 1995). Water deficits can alter water potential gradients and prevent host growth while not affecting pathogen growth. Water deficits affect host resistance by decreasing photosynthetic activity and protein activity that could decrease the synthesis of metabolites and enzymes important for disease resistance (Boyer, 1995). Disease-causing organisms are present around the plant and attack living tissues under particular conditions that weaken the host. Examples are found mostly among root rots, stem rots and vascular wilt. Water deficits in legumes lead to fewer photosynthates and a lower rate of nitrogen fixation (Boyer, 1995).

When studying water stress, both water deficits and excess water must be considered. Especially in Africa, the variations in precipitation and availability of moisture can bring about great changes in production. Excess water and flooding produce oxygen-deficiency, leading to the accumulation of toxic metabolites (Stolzky *et al.*, 1965 in Schoeneweiss, 1975), which interferes with host defences. Most root diseases are favoured by wet soils (Levitt, 1972). Land slope plays a role in the flooding of certain areas. In a survey, Agrawal (1989) found pigeonpea infected by *Phytopthora drechsleri* Tucker f. sp. *cajani* (Mahendra Pal, Grewal & Sarbhoy) Kannaiyan, Ribeiro, Erwin & Nene mainly in poorly drained fields in India. In lowland flooded fields, disease incidence was minimum in resistant cultivars and maximum in susceptible cultivars. In midland and upland conditions resistant and susceptible cultivars had the same disease incidence, suggesting that susceptible varieties can be grown successfully in well-drained conditions.

Light

Yarwood and Hooker (1966), in Schoeneweiss (1975), showed that light intensity and frequency could be a predisposing factor in many plant diseases. Singh and Chauhan (1986), as cited by Singh and Chauhan (1992), found that light and darkness affect lesion development, caused by *Ph. drechsleri* f.sp. *cajani,* on pigeonpea. The increase in lesion size is higher in darkness compared with continuous light in the glasshouse.

Presence of other parasites

According to Colhoun (1979) certain pathogens can alter the physiological environment within their hosts, and this has an effect on the host's reaction to other pathogens. Wounding by nematodes provide suitable points of entry to pathogenic fungi (Colhoun, 1979). A variety of antagonistic microorganisms coexist with pathogens in soil (Agrios, 1997), causing an environment of starvation or of toxic metabolites. Upadhyay and Rai (1987) showed the inhibition of *F. udum* by substances from other microorganisms. Volatile substances emanating from *Penicillium rubrum* Stoll showed the highest inhibition of radial growth of *F. udum* by 24 % after 48 h but stimulated by the same after 120 h of incubation. Furthermore, the rhizosphere soil of healthy pigeonpea plants showed inhibitory effect on the pathogen due to the presence of some antagonists, with *Aspergillus niger* Van Tieghem having the greatest suppressing ability. Microbial antagonism is an important factor for biological control of soil-borne pathogens (Garrett, 1965 in Upadhyay and Rai, 1987) affecting the establishment of the pathogen in the rhizosphere. Gaur and Sharma (1991) tested microorganisms isolated from the rhizosphere soil of pigeonpeas for antagonistic action towards *F. udum*. *Trichoderma viride* Pers. was most effective in controlling the disease, followed by *A. niger*, *Streptomyces* sp. and *Penicillium* sp.

Soil pH/soil types

Singh (1999) studied the effect of soil types and some minerals on development of wilt disease caused by *F. udum* on pigeonpea. Maximum mortality occurred in the loam soil and similarly, acidic soil showed maximum disease

control. Calcium sulfate caused the maximum decline of the disease. Upadhyay and Rai (1987) found incidence of wilt disease of pigeonpea to be favoured by slightly acidic to slightly alkaline soils containing 50 % or more sand particles. Optimal soil pH of 6.2-7.0 is important for root nodulation of soybean plants (Sinclair, 1999). Acidic soil can accentuate damage from Sclerotium blight, whereas sub-optimal soil pH, which reduces nutrient availability, increases the damage from a number of pathogens.

Altitude

Kumar, Dutta and Prasad (1991) found that altitude influenced disease incidence of pigeonpea diseases. Powdery mildew was predominant at higher altitudes during the rainy season and *F. udum* was the major disease at low altitudes. Leaf spot and blight diseases caused by *Phoma, Botrytis, Colletotrichum, Cercospora* and *Alternaria* spp. were more prevalent and severe at higher elevations.

Cultural practices

Most vegetable growers prefer to plant on a smooth seed bed free of debris (Sumner *et al.*, 1995). With moldboard plowing, the populations of *R. solani* in topsoil are reduced as well as root diseases in many agronomic crop-vegetable rotations. Concern about soil erosion favours conservation tillage as an alternative to conventional moldboard plowing (Sumner *et al.*, 1986). Conservation tillage includes any planting system that retains at least 30 % residue cover on the soil surface after planting (Hiemstra and Bauder, 1984 in Sumner *et al.*, 1995). After applying relay inter-cropping, root and hypocotyl diseases, caused by *R. solani*, were more severe on snap bean seedlings following legumes-cucumber than legume-grass mixtures and cucumber (Sumner *et al.*, 1995), whereas disease severity on snap bean seedlings in plots following crucifer-cucumber was intermediate.

Diseases increase or decrease with the choice of crops for rotations. Pande *et al.* (1993) evaluated the effect of groundnut/pigeonpea inter-cropping on the incidence of diseases and yield components of groundnut. Inter-cropping

pigeonpea with groundnut enhanced the incidence of foliar fungal diseases, mainly rust (*Pu. arachidis*) and late leaf spot (*Phaeoisariopsis personata (*Berk. & M.A. Curtis) Arx [*Mycosphaerella berkeleyi* W.A. Jenkins]) in groundnuts. Higher yield was obtained in groundnut crops than in inter-cropped plots. Rao and Mathuva (2000) found that pigeonpea experienced higher wilt (15-25 %), caused by *F. udum*, in continuously grown pigeonpea/maize intercrop systems than in pigeonpea-maize (two season long duration break) rotation (3-19 %).

Wilt disease of pigeonpea was significantly suppressed in solarized soil in India (Singh, Jariwala and Rai, 1996). The presence of the antagonist *Aspergillus niger* or carbendazim pronounced the effect in solarized soil. Solarization by covering soil with clear transparent polythene sheets for six weeks during the summer season caused an 8 ˚C increase of soil temperature with conservation of 5 % moisture, compared to the control (Rao and Krishnappa, 1995). The latter conditions lead to a significant reduction in *F. oxysporum* f. sp. *ciceris* (80.8 %) and weed (80.6 %) population densities. Availability of soil nutrients was increased without detrimental effect on soil characteristics.

CONCLUDING REMARKS

It is clear from the present review that the three components of the disease triangle strongly influence disease occurrence and development in legume pathosystems. Disease results from the interaction between the host, pathogen and environment, and a change in any one of these components, brings about a concomitant change in one or more of the other components. Environmental influences can be of advantage or disadvantage to the host and/or the pathogen. Detrimental effects on the host can be limited to a certain extent by choice of cultivation locality. This may be favourable for the host, but all potential pathogens cannot be excluded from a certain cultivation locality. Many pathogens have adapted to survive in conditions, similar to those which are optimal for development of the host plant. Often, despite efforts to control a certain disease, the pathogen adapts to the new conditions, for example overcoming newly introduced host

resistance.

This review has shown that pathogens attacking legumes, display high host-specificity and have unique individual requirements. Sub-components of each of the disease triangle components, can be manipulated to shift the balance of the whole system, towards better disease management.

Cultivation of a new legume crop is aimed at making available a valuable alternative food source, without incurring high costs. This will have an impact on the choice of cultural practices that can be applied, with fungicides being effective in controlling disease in most cases, but unfortunately too expensive.

Host resistance offers a lasting form of disease control, even if plant breeding is a time-consuming practise. Intercropping legumes with other crops has proven to be a valuable practise, not only providing more income from the extra crop, but enriching the soil as well. Also, intercropping can be used to control disease, depending on choice of crops. When considering an integrated pest management programme, integrated control measures are used towards successful cultivation of a crop, keeping disease at an acceptable level. A holistic perspective of pigeonpea diseases, that includes consideration of actions taken regarding all sub-components, will greatly assist management decisions.

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CHAPTER 2

CHARACTERIZATION OF *CERCOSPORA APII* AND *PASSALORA CAJANI* ISOLATES ASSOCIATED WITH PIGEONPEA IN SOUTH AFRICA

INTRODUCTION

Pigeonpea (*Cajanus cajan* (L.) Millsp.) is grown in several countries in Eastern and Southern Africa, the main producers being Kenya, Malawi, Mozambique, Tanzania and Uganda (Kimani, 2001). Pigeonpea is a new crop in South Africa, being considered for commercial cultivation. Possible yield-limiting factors, such as pests and diseases, therefore need to be investigated.

In humid regions, leaf spot, caused by *Passalora cajani* (Hennings) U. Braun & Crous (syn. *Mycovellosiella cajani* (Hennings) Rangel ex Trotter; syn. *Cercospora cajani* Hennings) (Anonymous (a); Crous and Braun, 2003) can cause yield losses up to 85 % in *C. cajan* (Reddy *et al.*, 1993). Warm (25 °C), humid weather favours the disease (Reddy *et al.*, 1993). Symptoms first appear as small circular to irregular necrotic spots or lesions, usually on older leaves. These lesions coalesce, causing leaf blight and defoliation. Symptoms similar to these were observed near Nelspruit, in the Mpumalanga province of South Africa during 2001.

The genus *Cercospora* represents some very destructive plant pathogens (Goodwin, Dunkle and Zismann, 2001), occurring on a wide range of crops (Pollack, 1987 in Goodwin *et al.*, 2001). Many attempts have been made to clarify the taxonomy of the genus (Deighton, 1967, 1973, and 1976 in Goodwin *et al.*, 2001). Culture and temperature requirements have been studied for several *Cercospora* spp. occurring on legumes. Martin and Walters (1982) investigated optimum dew temperature for *Cercospora kikuchii* (Matsumoto & Tomoyasu) M. W. Gardner infection of soybeans. Vathakos and Walters (1979) evaluated various culture media for their ability to support sporulation of *Ce. kikuchii.* Wadia and Butler (1994) studied the relationship between temperature and latent periods of leaf spot, caused by *Cercospora arachidicola* Hori on groundnut (*Arachis hypogaea* L.).

Molecular studies have proven useful in resolving relationships (Gielly and Taberlet, 1996) between fungal pathogens of plants (Berbee, 2001). Goodwin *et al.* (2001) performed a large-scale phylogenetic analysis to test hypotheses about the evolutionary history of *Cercospora* and *Mycosphaerella*, contributing to

resolution of taxonomic confusion surrounding the genus and adding new knowledge regarding the classification of newly isolated and known pathogens.

The prime objective of this study was to characterize the fungal pathogens associated with pigeonpea leaf spot in plantings near Nelspruit in terms of sensitivity to fungicides *in vitro*. Optimum temperature and nutritional requirements *in vitro* were also studied as a secondary objective. Molecular analysis was applied to corroborate the results of cultural studies.

MATERIALS AND METHODS

Isolate maintenance

Eleven fungal isolates (Table 2.1) were isolated from diseased plant material obtained from the Nelspruit area, in Mpumalanga, South Africa during 2001. Pigeonpea leaves, with leaf spot, were surface disinfected by spraying with 70 % ethanol (to break surface tension), followed by soaking in 1.75 % sodium hypochlorite (NaOCl) for 1 min and rinsing three times in sterile, distilled water (Baxter & Van der Linde, 1999). Pieces of leaves (4 mm^2) were then plated aseptically onto corn meal agar (17 g CMA, Oxoid®, Basingstoke, Hampshire, England). All media contained 0.3 ml streptomycin sulphate (Novo-Strep, Novo Nordisk [Pty] Ltd., Johannesburg)/L to inhibit bacterial growth. After 3 days of growth at 25 °C, isolations were made on potato dextrose agar (39 g PDA, Biolab®, Wadeville, Gauteng, South Africa) plates. Isolates were maintained on PDA agar slants and on agar plugs in sterile, distilled water at 4 °C. All eleven isolates were identified at the Centraalbureau voor Schimmelcultures in the Netherlands (Table 2.1), and subsequently deposited into their collection.

Inoculum production

The 11 isolates, three of *Ce. apii* and eight of *P. cajani*, were first grown on Petri dishes (90 mm) containing 25 ml V-8 juice agar (100 ml V-8 vegetable juice, Campbell Soup Company, U.S.A., 3 g $CaCO_3$, and 20 g/L water agar, pH 6), and incubated at 25 °C (Orth and Schuh, 1994). Ten-day-old cultures were induced to

sporulate by placing them in a sporulation room for 12 h, under black light (near-UV light), at 25 ˚C. Mycelium from isolates were then transferred to PDA plates and grown for 10 days at 25 ˚C before use.

Pathogenicity tests

To determine whether the 11 isolates (Table 2.1) were pathogenic, several pathogenicity tests were done.

Plant cultivation. Seeds of three medium-duration (MD) (ICP 6927, ICPL 87119 and ICPL 87051) and three long-duration (LD) (ICEAP 0053, ICEAP 0040, ICEAP 0020) pigeonpea varieties were obtained from the Lowveld Research Unit, Mpumalanga. Four seeds of each variety were planted in each of three replicate pots (300 ml) containing a steam-sterilized mixture of soil-peat moss (1:1 v/v). Plants were grown at 18-25 ˚C in a disease free, air-conditioned glasshouse cubicle. Once seedlings reached the V1-growth stage, plants were thinned to two per pot and 50 ml of 10 g/L hydroponic nutrient solution (3:2:1 N:P:K) was added as a weekly soil drench to each pot.

Detached leaf assay. Pigeonpea leaves from 1-month-old plants were collected from the greenhouse, surface disinfected by spraying with 70 % ethanol, followed by soaking in 1.75 % NaOCl for 1 min and rinsed three times in sterile, distilled water. The leaves were then placed in square bio-assay dishes (245 x 245 x 25 mm) (© A/S Nunc, Roskilde, Denmark), containing autoclaved blotting paper, 12 leaves per dish. A 5 mm^2 PDA piece, taken from an actively growing colony edge, was placed asceptically, mycelium side down on each of four leaves (two leaves adaxial, and the other two abaxial side up) per isolate. For the control, 5 mm^2 PDA piece without any fungal growth on it, was placed asceptically onto the leaves. The dishes were placed in an incubator at 25 ˚C and inspected every three days for lesion formation. Dishes were kept moist by adding sterilized water with a micropipette.

The experiment was repeated, with a few adjustments to the layout. Four leaves, from six-month-old plants, surface disinfected as mentioned above, were placed in autoclaved glass Petri dishes containing filter paper for each isolate. Inoculum was prepared for each isolate from colonies, grown on PDA plates as for

inoculum production, by adding 5 ml sterilised water to each PDA dish, and detaching conidia with a glass rod. Inoculum was adjusted to a spore suspension of 1 x 10^5 spores ml^{-1} (Ahmed, 2002). Five µl of the inoculum (Clulow, Lewis and Matthews, 1991) was placed on each of three leaves (adaxial or abaxial side randomly selected) per isolate and incubated at 25 ˚C. The control consisted of placing 5 µl of distilled water on leaves. Leaves were inspected every three days for lesion formation, and dishes were kept moist by adding sterilised water with a micropipette.

Whole plant tests. Two inoculum mixtures were prepared, one containing the *P. cajani* isolates and another containing the *Ce. apii* isolates, using the above-mentioned method. Six six-month-old and six three-month-old plants, per mixture, were inoculated using an atomizer. Plants were atomized to run-off and left to dry off at 25 ˚C. For the first experiment, the inoculated plants were placed in a dew chamber for 24 h (Orth and Schuh, 1994), allowed to dry off and moved to the greenhouse. In the second experiment, the inoculated plants were placed in a dew chamber for five consecutive nights of 16 h and allowed to dry during the day, bringing the total period of intermittent leaf wetness to 80 h (Wadia and Butler, 1994). Plants were then moved to a greenhouse cubicle with a temperature of 18-25 ˚C. Plants were inspected every three days, over a period of one month, for lesion development.

Effect of medium on growth

A 5-mm-diameter plug of each isolate, grown on PDA for 10 days, was removed from the edge of an actively growing colony with a cork-borer and placed aseptically, mycelium side down in the centre of each Petri dish, respectively containing 25 ml of V-8 juice agar, CMA, PDA and malt extract agar (MEA, 15 g water agar + 20 g ME, Biolab®, Wadeville, Gauteng, South Africa). The plates were incubated at 25 ˚C. Each treatment was replicated three times. Colony diameter was determined by calculating the mean of two perpendicular measurements from the centre of the colony on each plate after 10 days. The experiment was conducted twice and data from the first and second experiments were pooled if experiments, according to analysis of variance, were similar.

Variances were tested for homogeneity using Bartlett's test (Bartlett, 1937).

Effect of temperature on growth

A 5-mm-diameter agar plug from a 10-day-old isolate, grown on PDA, was removed from the actively growing edge of the colony with a cork-borer and placed aseptically, mycelium side down, in the centre of each Petri dish, containing 25 ml PDA and incubated at 15, 20 25 and 30 ˚C. Each treatment was replicated three times. Radial colony growth was calculated after 10 days by determining the mean of the two perpendicular measurements of the colony diameter on each plate. The experiment was conducted twice and data from the first and second experiments were pooled if experiments, according to analysis of variance, were similar. Variances were tested for homogeneity using Bartlett's test (Bartlett, 1937)

***In vitro* efficiency of fungicides**

Autoclaved PDA was cooled to 45 ˚C and amended with 0.3 ml/L streptomycin sulphate and with the following fungicides: flusilazole/carbendazim (Punch-Xtra® - 125 g +250 g/L SC), propiconazole (Tilt® - 250 g/L EC), mancozeb (Dithane M-45® - 800 g/kg WP), chlorothalonil (Bravo® - 500 g/L SC) and azoxystrobin (Amistar® - 500 g/L SC). All five fungicides are registered against Cercospora leaf spot (Thomson, 1991). The fungicides were added to the PDA in the following concentrations: 0.5, 0.1, 1, 5, 10, 25 and 50 µg a.i./ml, and unamended PDA served as the control. A 5-mm-diameter plug was then removed from an actively growing colony of each isolate with a cork-borer and placed mycelium side down onto the plates containing the fungicide amended agar. Plates were incubated at 25 ˚C for 10 days and radial colony growth determined by calculating the mean of the two perpendicular measurements of the colony diameter on each plate. Each treatment had three replications and the experiment was conducted twice. The inhibition percentage was plotted for each isolate, at each of the concentrations for each fungicide. Only the three fastest growing isolates of each genus were used in above-mentioned experiment, namely STE-U 4567, 5250 and 10456 of *P. cajani* and STE-U 5325, 5326 and 5327 of *Ce. apii*.

Cluster analysis

An investigation was conducted using data obtained from culture media, temperature and fungicide studies, respectively, to determine whether physiological characteristics were consistent with molecular relationships between the genera. Cluster analysis was performed using the statistical program NCSS 2000 (BMDP Statistical software Inc., Los Angeles, California, U.S.A.), using group average (Unweighted Pair-Group).

DNA isolation, sequencing and phylogeny

Genomic DNA was isolated from fungal mycelium of the 11 isolates (Table 2.1), grown on MEA plates (Lee and Taylor, 1990). The primers ITS1 and ITS4 were used to amplify part (ITS) of the nuclear rRNA operon spanning the 3' end of the 18S rRNA gene, the first internal transcribed spacer (ITS1), the 5.8S rRNA gene, the second ITS region and the 5' end of the 28S rRNA gene. The PCR reaction mixture used consisted of 0.75 units Biotaq (Bioline, London, UK), 1x PCR buffer, 1.5 mM $MgCl_2$, 0.2 µM of each dNTP, 5 pmoles of each primer, approximately 10-30 ng of fungal genomic DNA and was made up to a total volume of 25 µL with sterile water. Reactions were performed on a GeneAmp PCR System 2700 (Applied Biosystems, Foster City, CA) and the cycling conditions consisted of denaturation for 5 min at 96 °C, followed by 30 cycles at 96 °C (30 s), 55 °C (30 s), 72 °C (90 s) and a final 7 min extension step at 72 °C to complete the reaction. To test whether the isolates were identical, part of the elongation factor 1-alpha (EF) gene was amplified with primers EF1-728F and EF1-986R (Carbone and Kohn, 1999). The same PCR conditions were used for EF-1a as for ITS. PCR products were separated by electrophoresis at 80 V for 1 h in a 0.8 % (w/v) agarose gel in 0.5x TAE running buffer (0.4 M Tris, 0.05 M NaAc, and 0.01 M EDTA, pH 7.85) and visualised under UV light using a GeneGenius Gel Documentation and Analysis System (Syngene, Cambridge, UK), following ethidium bromide staining. The amplification products were purified according to the manufacturer's instructions using a commercial kit (GFX PCR DNA and Gel Band Purification Kit, Amersham Pharmacia Biotech Europe GmbH, Germany). Sequencing reactions were carried out using the PCR primers in ABI PRISM Big Dye Terminator Cycle

v3.0 Sequencing Ready Reaction Kit (Applied Biosystems) according to the manufacturer's recommendations. The reaction was analysed on an ABI Prism 3100 Genetic Analyser (Applied Biosystems).

The ITS nucleotide sequences generated in this present study were added to other sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov) and the alignment was assembled using Sequence Alignment Editor v2.0a11 (Rambaut, 2002). The phylogenetic analyses of sequence data were done using PAUP (Phylogenetic Analysis Using Parsimony) version 4.0b10 (Swofford, 2001). Alignment gaps were treated as a fifth character state and all characters were unordered and of equal weight for the parsimony analysis. Maximum parsimony analysis was performed for all data sets using the heuristic search option with 100 random taxon additions and tree bisection and reconstruction (TBR) as the branch swapping algorithm. Branches of zero length were collapsed and all multiple, equally parsimonious trees were saved. Other measures calculated included tree length, consistency index, retention index and rescaled consistency index (TL, CI, RI and RC, respectively). The robustness of the resulting phylogenetic trees was evaluated by 1000 bootstrap replications (Hillis and Bull, 1993) and the trees were printed with TreeView Version 1.6.6 (Page, 1996).

Data analysis

All data were analysed using NCSS 2000. Data were subjected to an analysis of variance (ANOVA) and Tukey Kramer's test to determine differences among means.

RESULTS AND DISCUSSION

The isolates were identified, based on morphology and molecular characteristics as *Passalora cajani* (Hennings) U. Braun & Crous and *Cercospora apii* Fresen *Ce. apii* has been reported as a pathogen of several plants (Anonymous (b)), but no reports on pigeonpea have been made in South Africa. Despite several experiments to prove pathogenicity of the isolates on six *C. cajan*

varieties, no lesion formation was observed. However, the above-mentioned fungi were obtained from 72 % of initial isolations made from field material. This percentage indicates recurrent association of the pathogens with diseased *C. cajan* leaves. The fact that pathogenicity could not be proven, possibly because insufficient time was allowed for symptom development, may be an indication that these species have a long latent period. Pathogenicity tests need to be repeated, including a wider range of environmental and host conditions, especially taking into account plant senescence. Sinclair and Cerkauskas (1996) observed a latent infection for *Ce. kikuchii*, cause of leaf spot in soybeans. Neither inoculated seedlings nor young plants showed symptoms, with *Ce. kikuchii* usually only inducing foliar symptoms at the time of seedset, regardless of environmental conditions. Similarly, symptoms due to *Colletotrichum* spp., cause of anthracnose on soybean, typically only appear during early reproductive stages (Manandhar and Hartman, 1999), often as a result of latent infections (Cerkauskas, 1988 in Lenné, 1992).

The 11 isolates studied varied significantly ($P < 0.05$) in their ability to utilize the four culture media. The three *Ce. apii* isolates grew slower on CMA than on any other medium (Fig. 2.1 B). Isolate STE-U 5325 grew fastest on PDA (34.1 mm^2), whereas STE-U 5326 and 5327 grew fastest on V-8 juice agar with radial colony diameters of 26.1 mm^2 and 29.1 mm^2 respectively. Most *P. cajani* isolates grew significantly slower on CMA, with STE-U 5335 and STE-U 5337 being the exceptions (Fig. 2.2 B). However, there was no significant ($P < 0.05$) difference in colony diameter between the four media for these isolates. Most *P. cajani* isolates grew fastest on V-8 juice agar. V-8 juice agar is a well known, preferred medium for cultivation of *Cercospora* spp. (Vathakos and Walters, 1979; Dhingra and Sinclair, 1985; Orth and Schuh, 1994). Sieber-Canavesi, Petrini and Sieber (1991) successfully used substrate utilization tests to distinguish between *Leptostroma* spp. which were morphologically indistinguishable.

A significant ($P < 0.05$) interaction between isolates and temperature occurred. A temperature of 25 °C was significantly ($P < 0.05$) more conducive to growth of all three *Ce. apii* isolates (Fig. 2.1 A), whereas least radial growth occurred at 30 °C. No growth was recorded for any of the *P. cajani* isolates at 30 °C (Fig. 2.2 A), whereas all isolates, excluding STE-U 5335, 5337 and 5338,

displayed optimum growth at 25 ˚C. STE-U 5335 and 5337 grew equally fast at 20 and 25 ˚C, and STE-U 5338 grew significantly ($P < 0.05$) faster at 20 ˚C than at the other temperatures. These findings are in agreement with Reddy *et al.* (1993) for other *P. cajani* isolates, the cause of pigeonpea leaf spot.

Inhibition by fungicides varied greatly with respect to the different isolates, different fungicides and fungicide concentration (Fig. 2.3-2.7). The general trend was that all five fungicides inhibited fungal growth effectively at concentrations higher than 5 µg a.i./ml. Reddy *et al.* (1993) suggested maneb (Indofil M 45®) as an effective fungicide against *Cercospora* spp. on pigeonpea. Onim (1980) in Kimani (2001) found that Dithane M-45 (80 % mancozeb) was most effective in controlling Passalora leaf spot, although not economical. In the present study, mancozeb was found to be least effective in inhibiting fungal growth of all the isolates. Flusilazole/ carbendazim inhibited fungal growth of *P. cajani*, as well as *Ce. apii* most effectively, with 40 % inhibition at 0.1 µl a.i./ml.,and inhibition percentage doubling at 0.5 µl a.i./ml for the *Ce. apii* isolates. However, Onim (1980) in Kimani (2001) reported that carbendazim caused slight stunting of plants and depressed grain yield in field trials.

Cluster analysis (Fig. 2.8) using fungicide data (illustrated in Fig. 2.3 - 2.7), showed STE-U 5250 to be polyphyletic to the other two *P. cajani* isolates, STE-U 4567 and 10456. The *Ce. apii* isolates (STE-U 5325, 5326 and 5327) were monophyletic to each other, and also shared monophyly with STE-U 4567 and 10456. From the results of medium and temperature experiments (Fig. 2.1 and 2.2), clustering revealed two groups, very dissimilar from each other (Fig. 2.9). One group contained all three *Ce. apii* isolates and the other group included all the *P. cajani* isolates, with little dissimilarities. The results of the physiological studies provided resolution of the phylogenitic relationships within the two isolated species. The two groups were the one containing the *Ce. apii* isolates, and the other the *P. cajani* isolates, as expected. Similarly, Goodwin *et al.* (2001), found a strongly supported monophyletic group that includes all of the true *Cercospora* species, this group being polyphyletic to *Passalora* spp. tested. *Cercospora apii* is classified as a true species (Stewart *et al*., 1999).

For ITS approximately 500 to 560 bases were determined for all isolates (Table 2.1), and approximately 300 bases for elongation factor 1- alpha. The

manually adjusted alignment of the ITS nucleotide sequences contained 39 taxa (including the two outgroups) and the EF alignment 14 taxa (including the two outgroups). The total alignment contained 968 characters including alignment gaps, of which 547 and 360 bases were used in the analysis of the ITS and EF regions respectively. Of the aligned 547 nucleotides for ITS, 222 characters were parsimony-informative, 48 variable characters were parsimony-uninformative and 277 were constant. The 360 aligned nucleotides of the EF regions consisted of 187 characters that were parsimony-informative, 91 variable characters were parsimony-uninformative and 82 were constant. Parsimony analysis of the ITS data yielded thirteen most parsimonious trees, one of which is shown in Fig. 2.10. Sequences obtained for the isolates formed two distinct clades, one of which was identified as *P. cajani* (100 % bootstrap support); while sequences of the other clade are found in *Cercospora* (72 % bootstrap support) and were identified as *Ce. apii*. Only a single most parsimonious tree was obtained from the EF data (Fig. 2.11) and in this tree the same two clades are found (both with bootstrap support values of 100%), with no variation present in either of the clades. Results of the molecular study are consistent with those of the physiological studies, concluded in two dendograms (Fig. 2.8 and Fig. 2.9). The subclustering formed in the *Cercospora* clade (Fig. 2.10) indicates some degree of evolutionary divergence in these specimens, corresponding to the results of the study done by Goodwin *et al.* (2001).

All the *P. cajani* isolates used in this study form a monophyletic group in both molecular and physiological studies, except in Fig. 2.8. In this dendogram the *P. cajani* isolates show a degree of polyphyly. This grouping is well supported and confirmed in Fig. 2.10 (ITS sequences), where the *P. cajani* isolates form a polyphyletic group with all the other *Passalora* specimens obtained from Genbank. This indicates that the *P.cajani* specimens are evolutionary more advanced than the *Ce. apii* specimens. No subclustering occurs in the *P. cajani* group, but the bootstrap value of 68 (Fig 2.10) indicates that there is a definite relation between the two monophyletic *Passalora* clades. However, a problem with ITS data is that it is not clear how many differences there are between closely related species (Goodwin *et al.*, 2001). It is not clear how closely related the specimens in these two clades are and therefore it is necessary to sequence more nuclear genes in

order to clarify the relationship between the two clades, using more isolates.

Fungal pathogens that attack legumes appear to have high host-specificity and unique individual physiological requirements (Colhoun, 1973; Huber, 1980). The latter characteristic made it possible in the present study to characterize the 11 isolates, associated with pigeonpea leaf spot, in terms of sensitivity to fungicides, optimum growth temperature and nutritional requirements, *in vitro*. The phylogenetic relationship of the two species to each other, and other closely related plant pathogens, was also determined by means of molecular analysis. The resulting classification supported and verified physiologically-based characterization. Although pathogenicity could not be shown for the isolates in the present study, it is probable that future studies concentrating on environmental and host factors will elucidate the underlying reason.

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Figure 2.1. A: Mean colony diameter of three isolates of *Cercospora apii* grown on potato dextrose agar at each of four temperatures: 15, 20, 25 and 30 °C. **B:** Mean colony diameter of three isolates of *Cercospora apii* grown on each of four culture media, incubated at 25 °C. Standard deviation is indicated by error bars.

Figure 2.2. A: Mean colony diameter of eight isolates of *Passalora cajani* grown on PDA at 15, 20, 25 and 30 ˚C. **B:** Mean colony diameter of eight isolates of *Passalora cajani* grown on four culture media, incubated at 25 ˚C. Standard deviation is indicated by error bars.

Figure 2.3. Percentage inhibition of radial colony growth by the active ingredient mancozeb for three isolates of *Passalora cajani* (**A**) and three isolates of *Cercospora apii* (**B**) at five different concentrations (0.1, 0.5, 1, 5 and 10 µg a.i./ml).

Figure 2.4. Percentage inhibition of radial colony growth by the active ingredient chlorothalonil for three isolates of *Passalora cajani* (**A**) and three isolates of *Cercospora apii* (**B**) at five different concentrations (0.1, 0.5, 1, 5 and 10 µg a.i./ml).

Figure 2.5. Percentage inhibition of radial colony growth by the active ingredient propiconazole for three isolates of *Passalora cajani* (**A**) and three isolates of *Cercospora apii* (**B**) at five different concentrations (0.1, 0.5, 1, 5 and 10 µg a.i./ml).

Figure 2.6. Percentage inhibition of radial colony growth by the active ingredients flusilazole/carbendazim for three isolates of *Passalora cajani* (**A**) and three isolates of *Cercospora apii* (**B**) at five different concentrations (0.1, 0.5, 1, 5 and 10 µg a.i./ml).

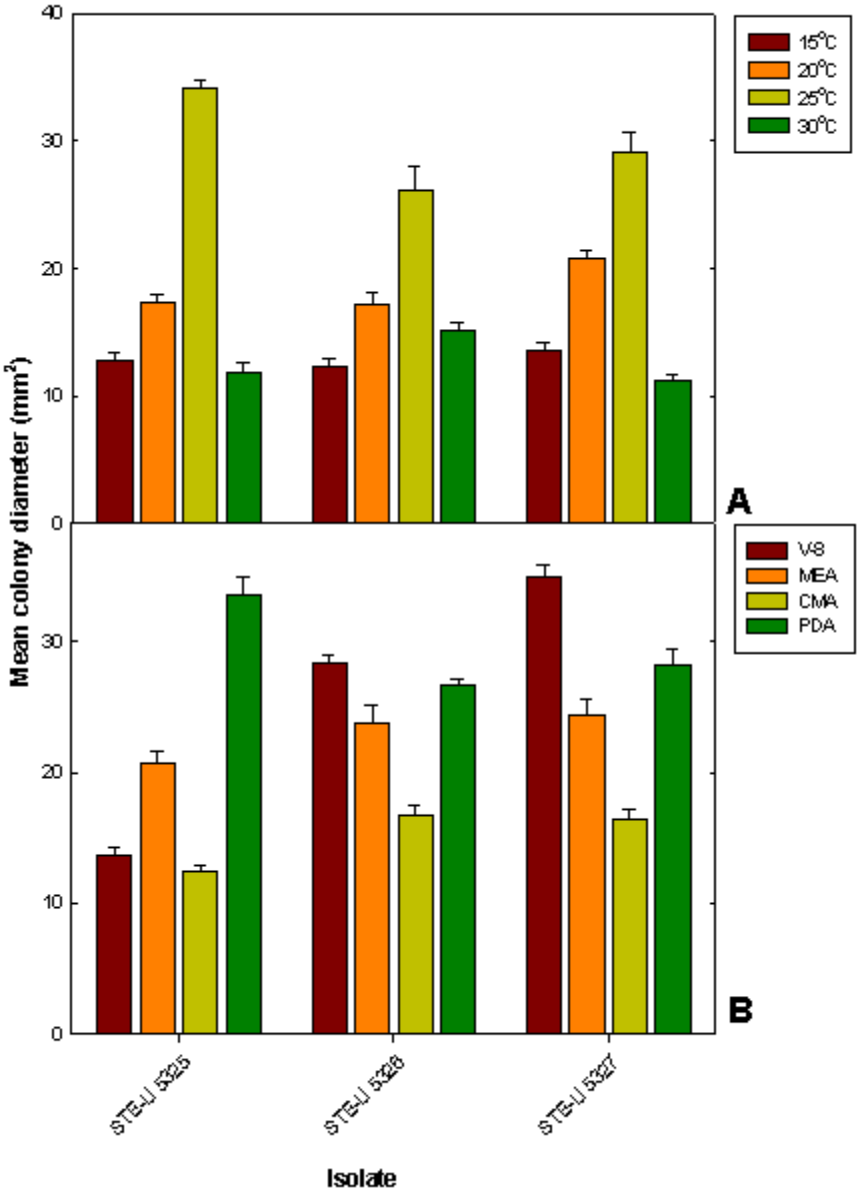
Figure 2.7. Percentage inhibition of radial colony growth by the active ingredient azoxystrobin for three isolates of *Passalora cajani* (**A**) and three isolates of *Cercospora apii* (**B**) at five different concentrations (0.1, 0.5, 1, 5 and 10 µg a.i./ml).

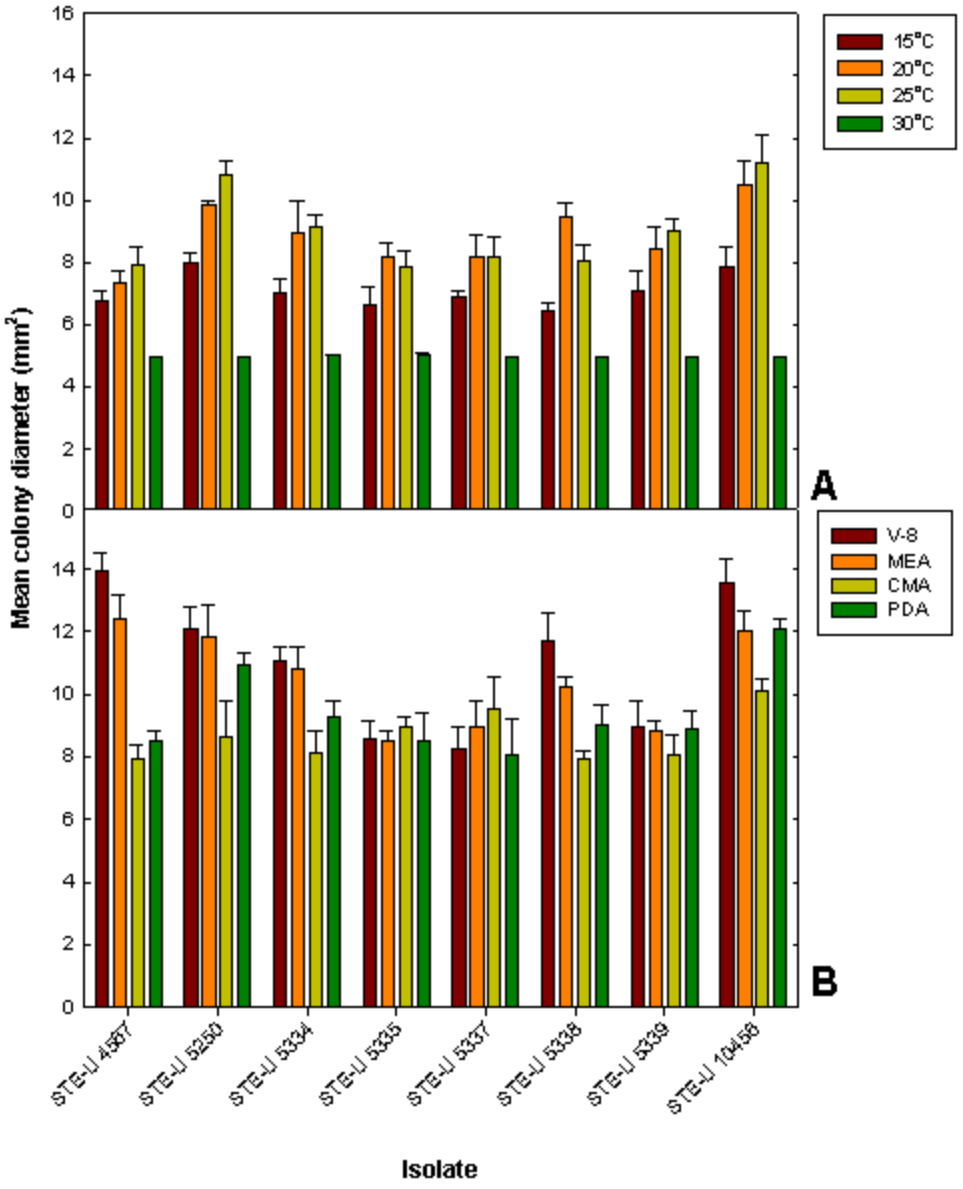
Figure 2.8. Cluster analysis based on inhibition percentages due to five fungicides for *Cercospora apii* and *Passalora cajani* isolates.

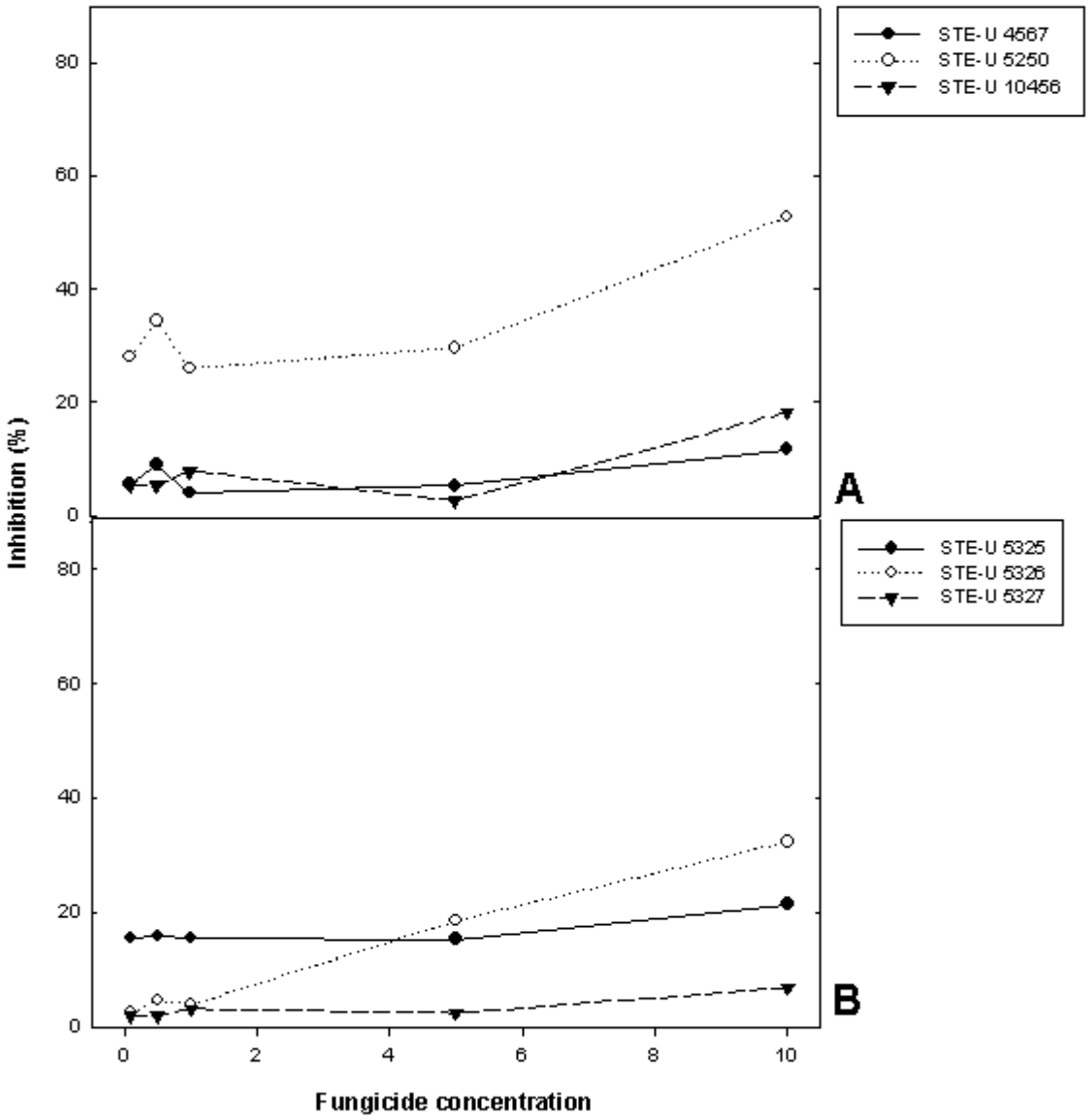
Figure 2.9. Cluster analysis based on colony diameters obtained on four culture media and at four temperatures for *Cercospora apii* and *Passalora cajani* isolates.

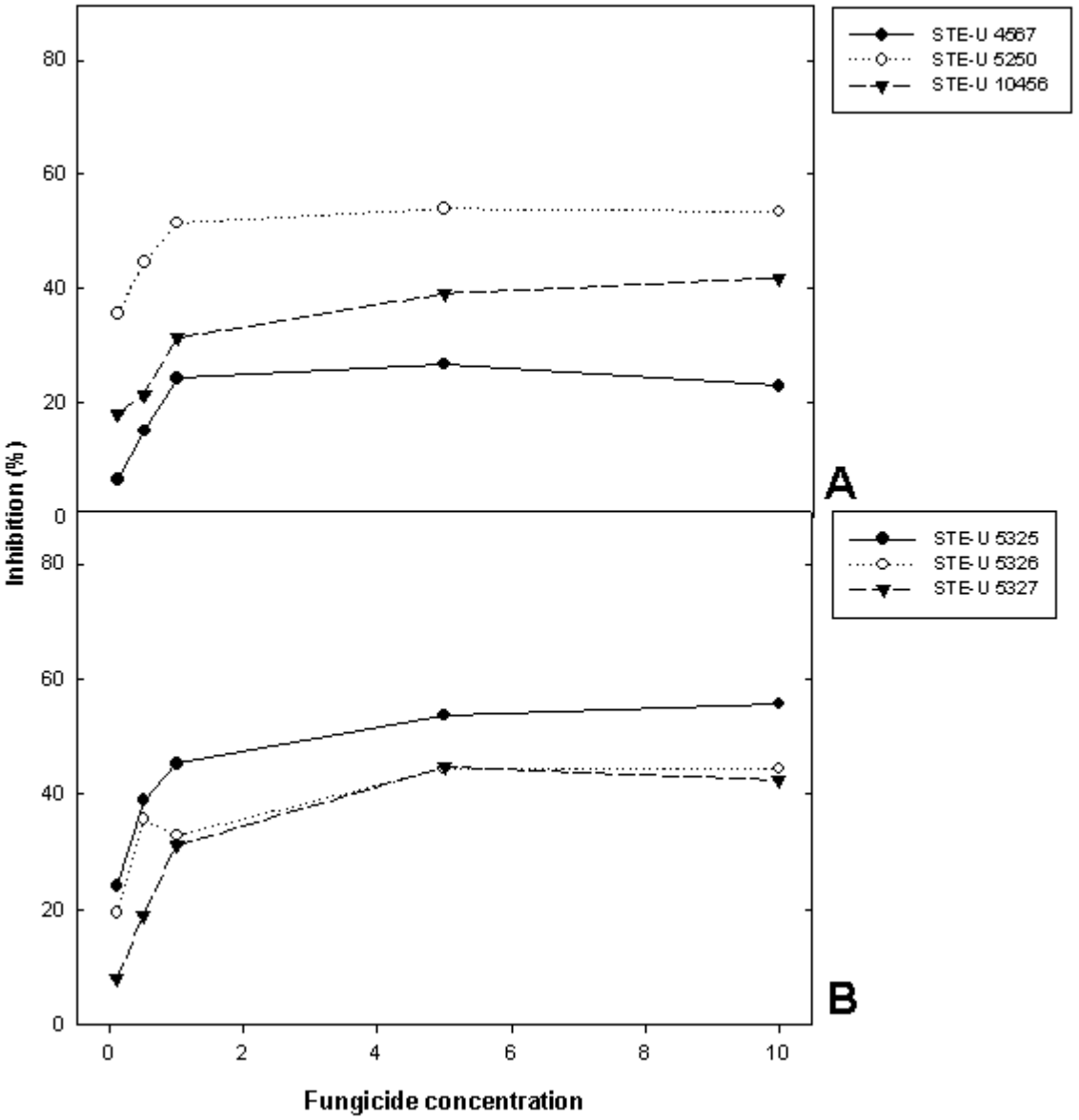
Figure 2.10. One of the thirteen most parsimonious trees obtained from ITS data. (TL = 574 steps, CI = 0.735, RI = 0.856, RC = 0.629). The scale bar indicates 10 changes and strict consensus branches are thickened. New sequences are shown in bold type. Bootstrap support from 1000 replicates are shown at the nodes. The tree was rooted in two *Phomopsis* species.

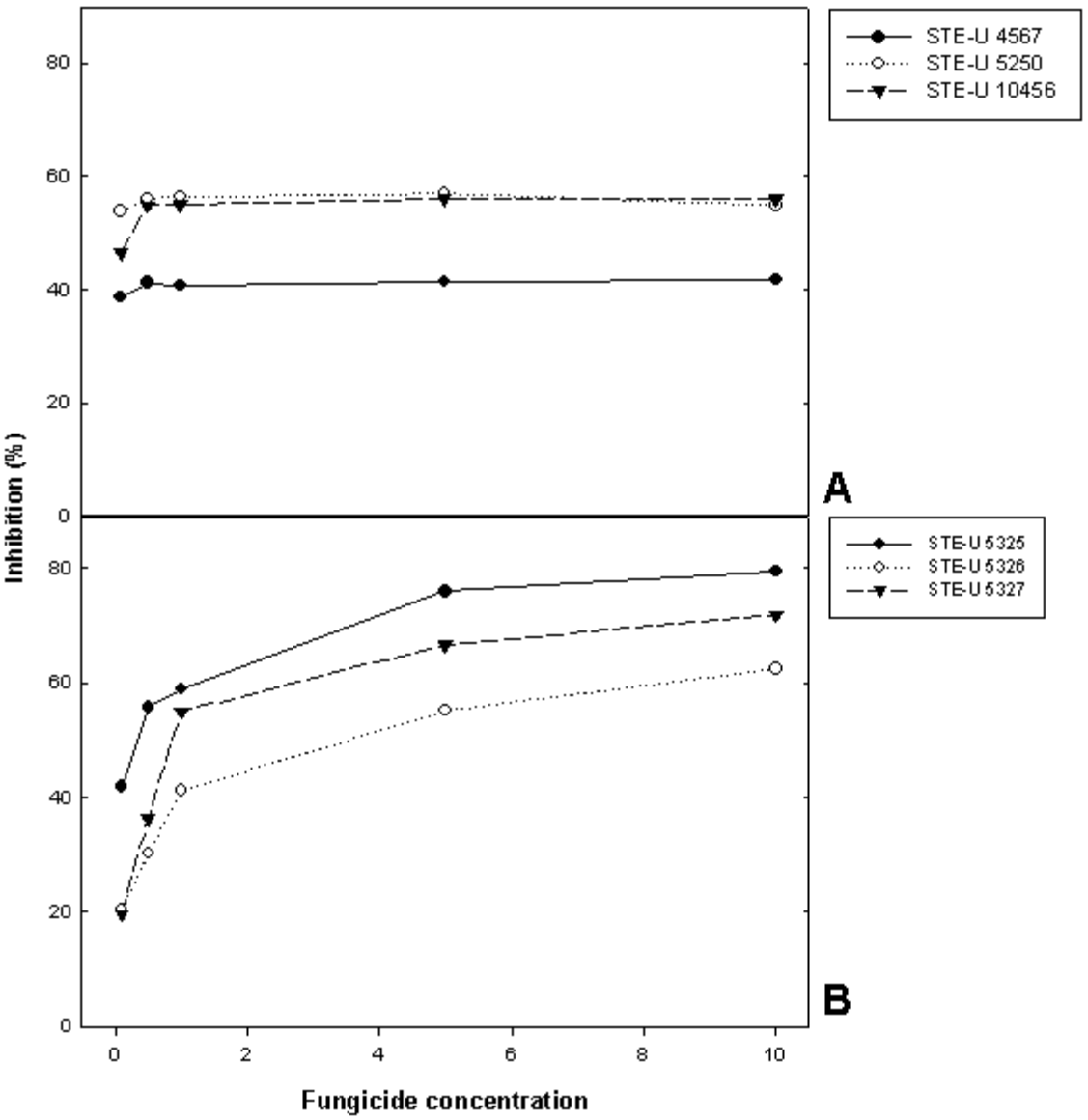
Figure 2.11. Single most parsimonious tree obtained from elongation factor 1-alpha data (TL = 393 steps, CI = 0.985, RI = 0.984, RC = 0.969). The scale bar indicates 10 changes. Bootstrap from 1000 replicates are shown at the nodes. The tree was rooted to two *Phomopsis* species.

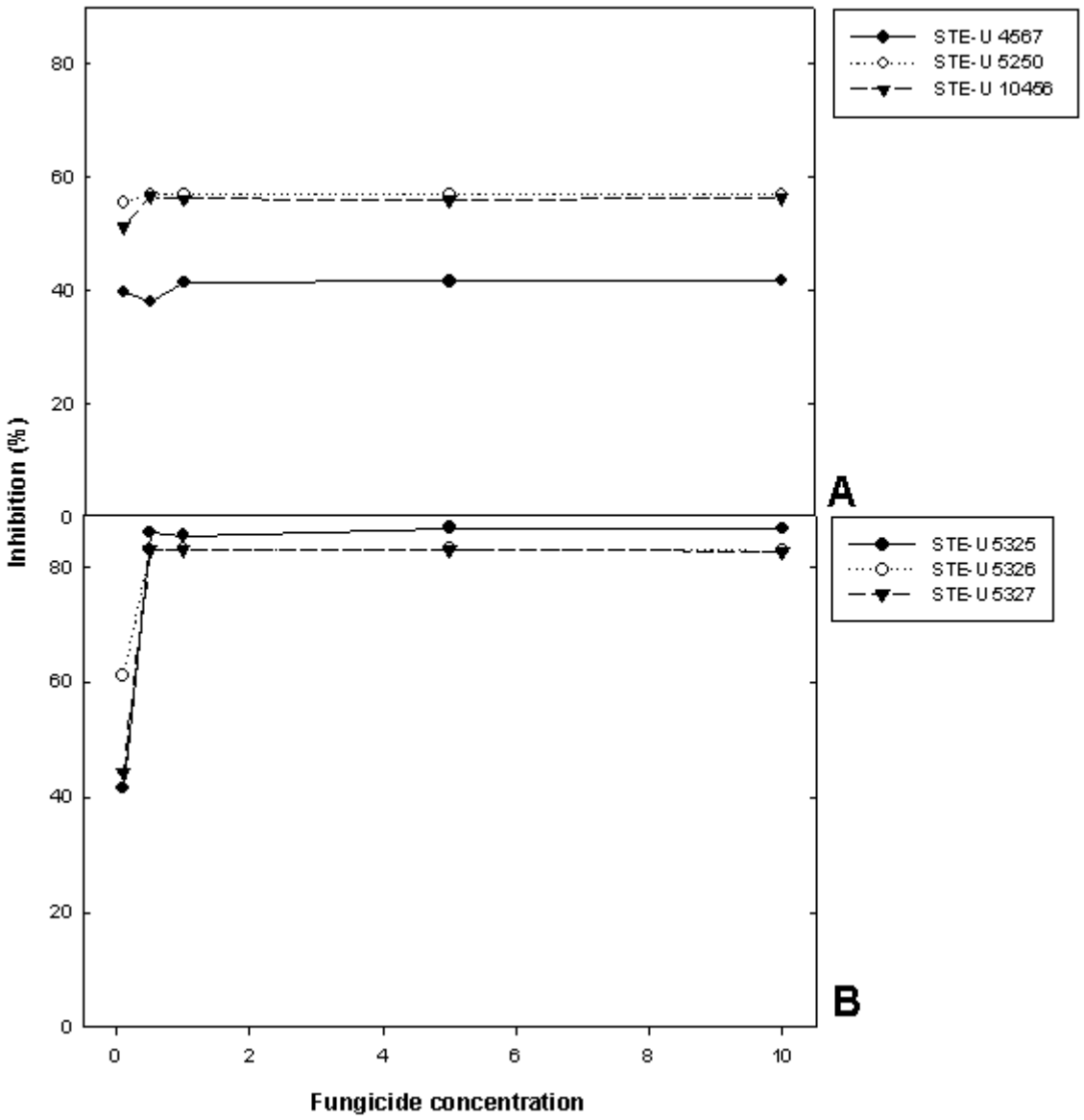

Mean colony diameter (mm²)
40
30
20
10
0
15°C
20°C
25°C
30°C
A
V8
MEA
CMA
PDA
B
STE-U 5325
STE-U 5326
STE-U 5327
Isolate


Mean colony diameter (mm²)
15°C
20°C
25°C
30°C
A
V-8
MEA
CMA
PDA
B
STE-U 4987
STE-U 5250
STE-U 5334
STE-U 5335
STE-U 5337
STE-U 5338
STE-U 5339
STE-U 10458
Isolate


STE-U 4567
STE-U 5250
STE-U 10456
STE-U 5325
STE-U 5326
STE-U 5327
A
B
Inhibition (%)
Fungicide concentration
0
20
40
60
80
0
2
4
6
8
10


STE-U 4567
STE-U 5250
STE-U 10456
STE-U 5325
STE-U 5326
STE-U 5327
Inhibition (%)
Fungicide concentration
A
B
0
20
40
60
80
0
2
4
6
8
10


STE-U 4567
STE-U 5250
STE-U 10456
STE-U 5325
STE-U 5326
STE-U 5327
A
B
Inhibition (%)
Fungicide concentration
0
20
40
60
80
0
2
4
6
8
10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STE-U 5250
STE-U 10466
STE-U 5325
STE-U 5326
STE-U 5327
A
B
Inhibition (%)
Fungicide concentration
0
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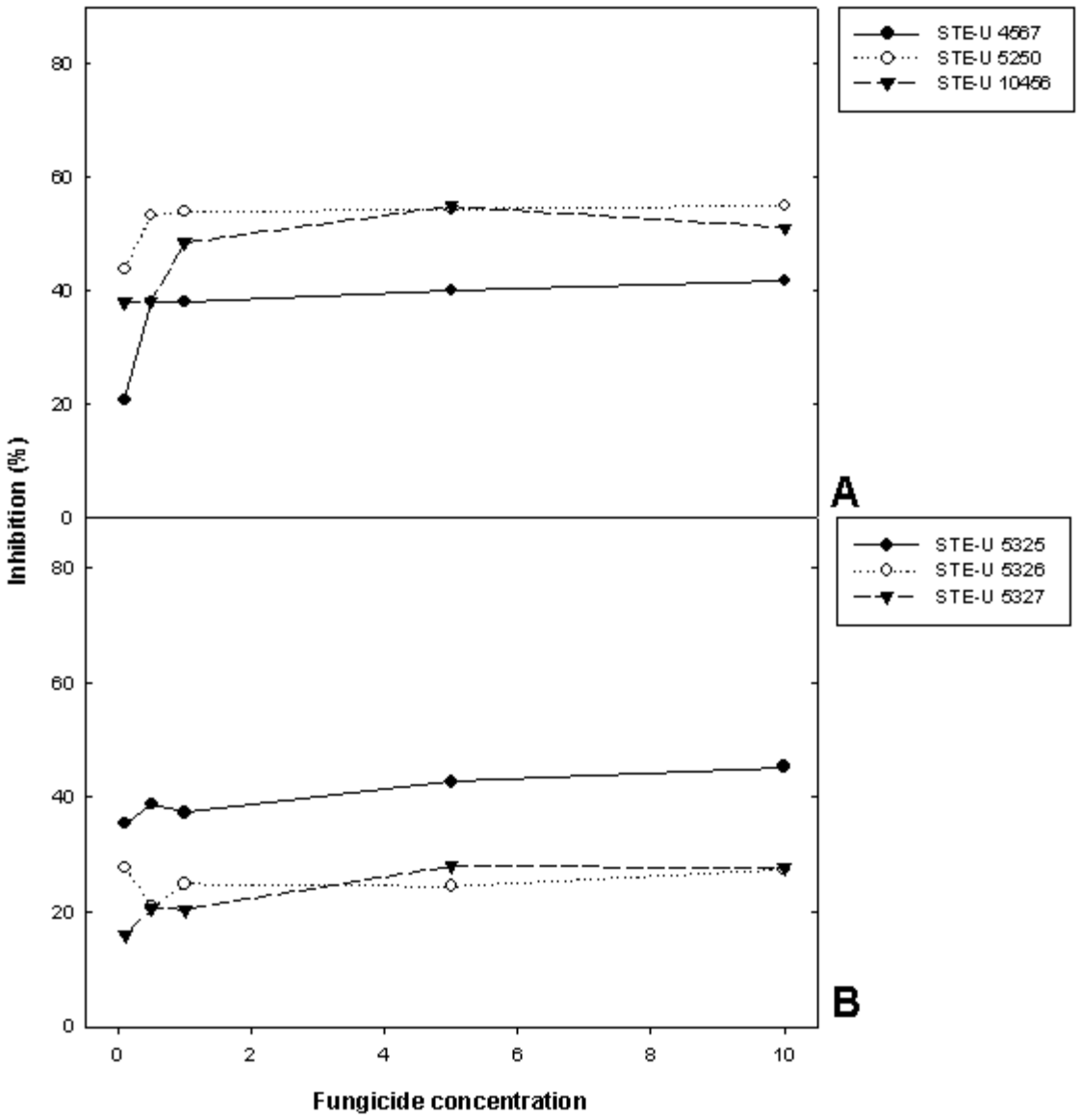
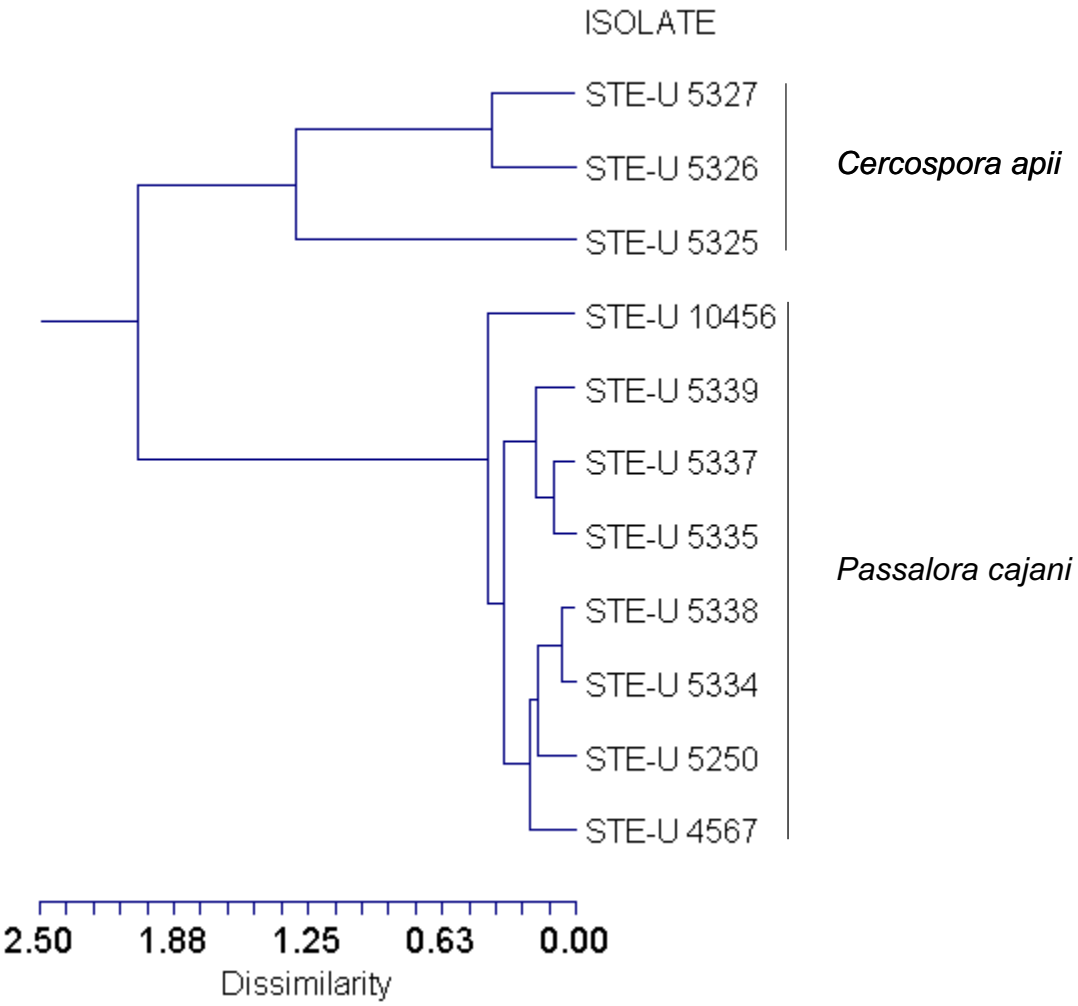
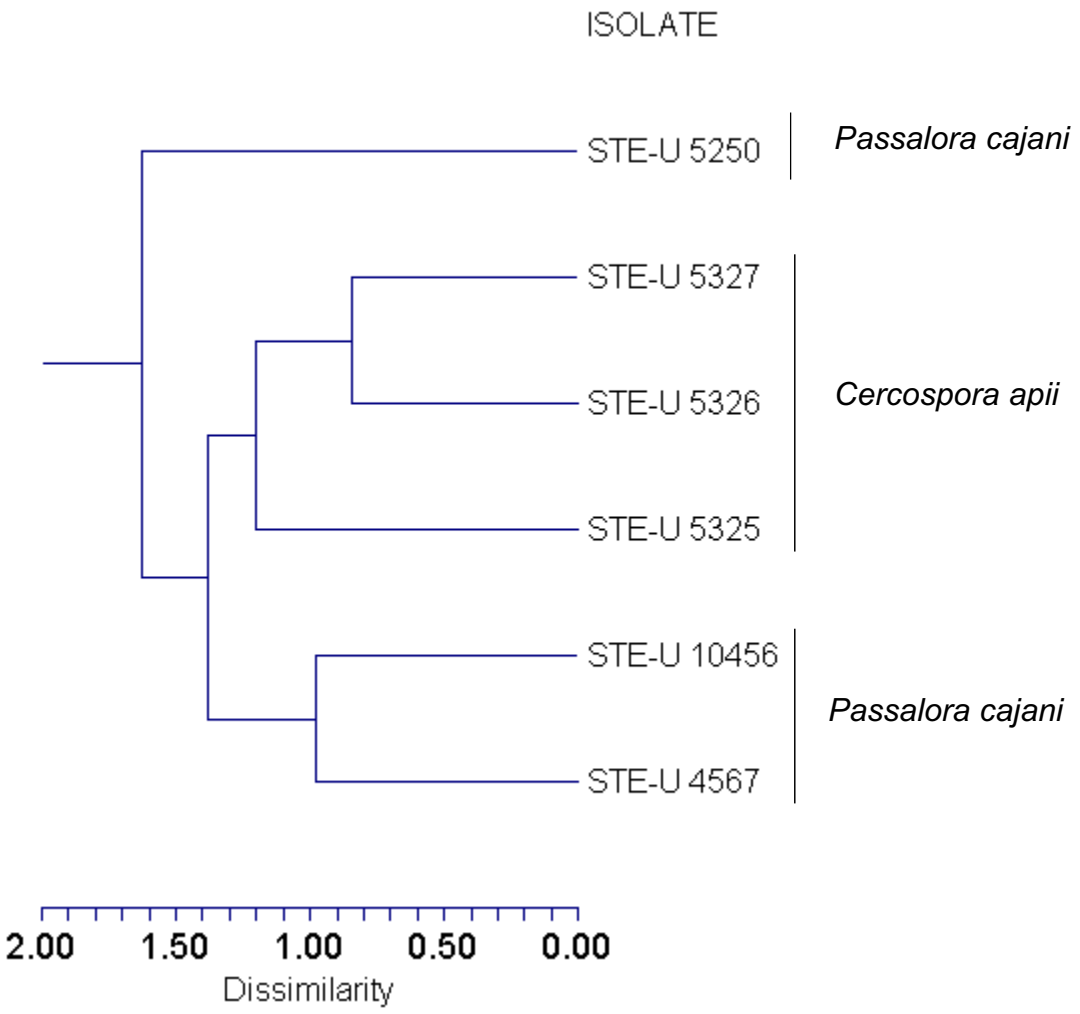
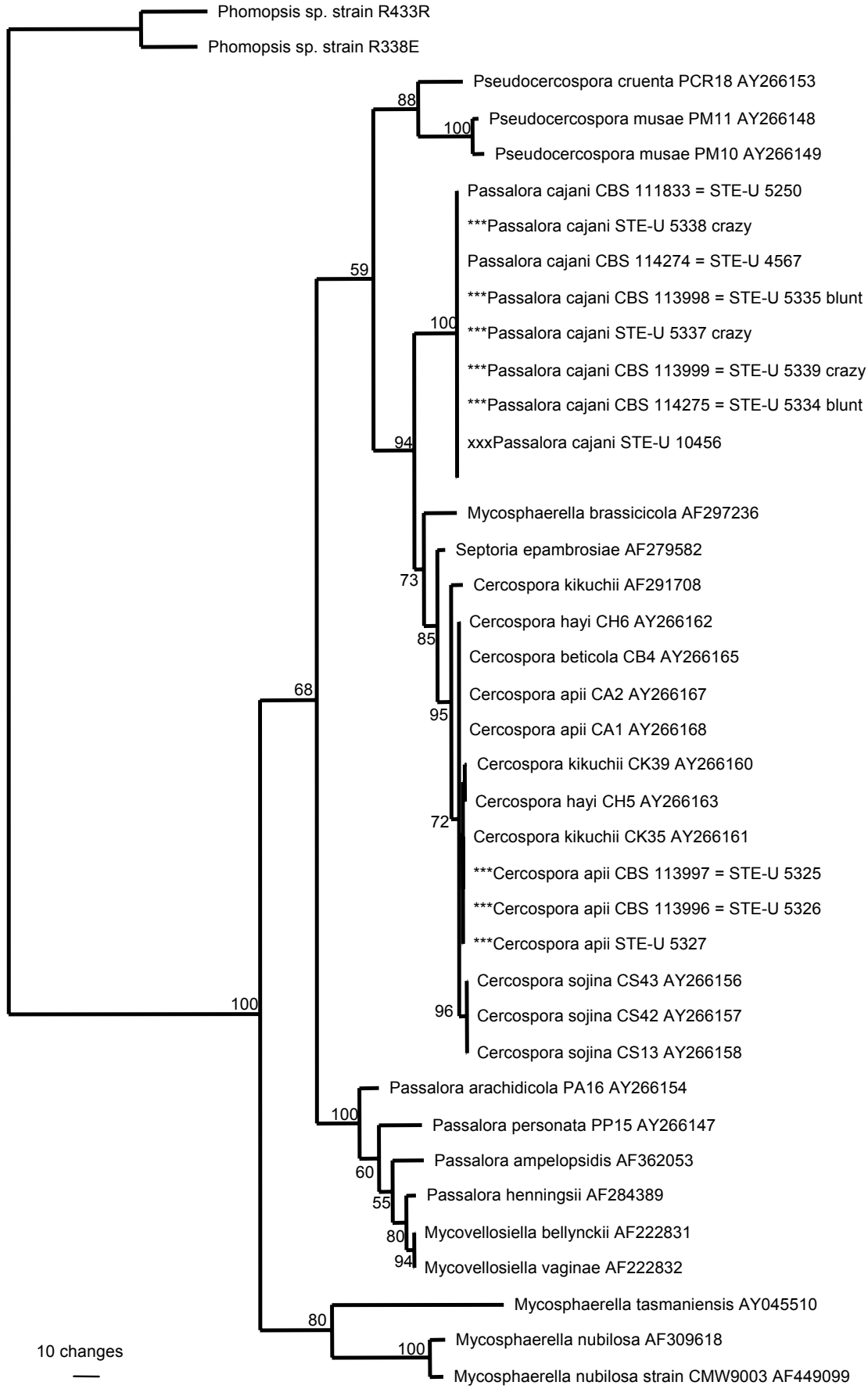
40
60
80
0
2
4
6
8
10

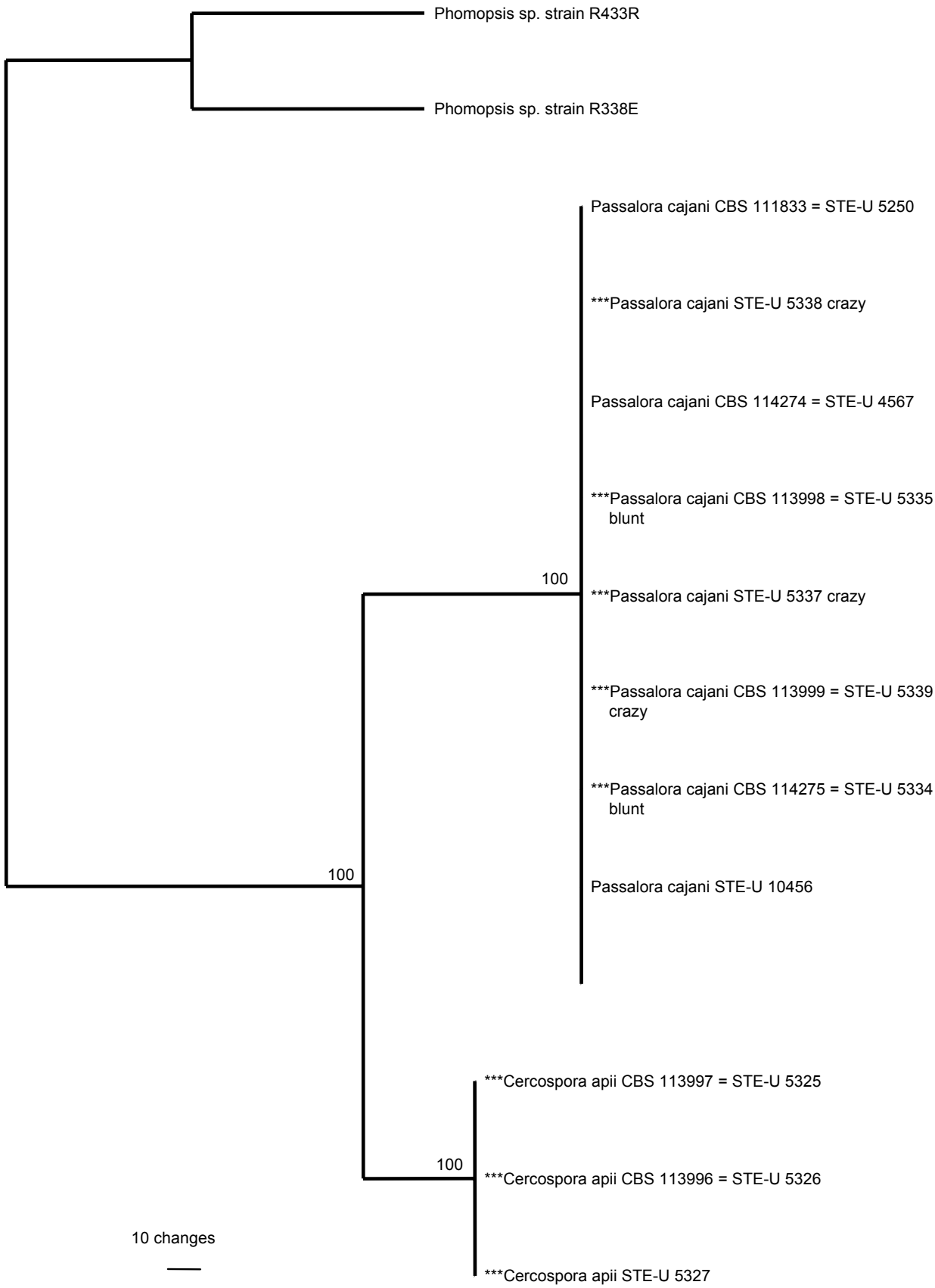

STE-U 4567
STE-U 5250
STE-U 10456
STE-U 5325
STE-U 5326
STE-U 5327
A
B
Inhibition (%)
Fungicide concentration
0
20
40
60
80
0
2
4
6
8
10


ISOLATE
STE-U 5327
STE-U 5326
STE-U 5325
STE-U 10456
STE-U 5339
STE-U 5337
STE-U 5335
STE-U 5338
STE-U 5334
STE-U 5250
STE-U 4567
Cercospora apii
Passalora cajani
2.50
1.88
1.25
0.63
0.00
Dissimilarity


ISOLATE
STE-U 5250
STE-U 5327
STE-U 5326
STE-U 5325
STE-U 10456
STE-U 4567
Passalora cajani
Cercospora apii
Passalora cajani
2.00
1.50
1.00
0.50
0.00
Dissimilarity



One of thirteen most parsimonious trees obtained from ITS data. (TL = 574 steps, CI = 0.735, RI = 0.856, RC = 0.629) . Bootstrap support from 1000 replicates are shown at the nodes. The tree was rooted to two *Phomopsis* species.



Single most parsimonious tree obtained from elongation factor 1-alpha data (TL = 393 steps, CI = 0.985, RI = 0.984, RC = 0.969) . Bootstrap support from 1000 replicates are shown at the nodes. The tree was rooted to two *Phomopsis* species.

Table 2.1 Culture numbers and names of fungi collected in Mpumalanga

Culture number	Fungus name
STE-U 5325/ CBS113997	*Cercospora apii* s.lat.
STE-U 5326/ CBS113996	*Cercospora apii* s.lat.
STE-U 5327	*Cercospora apii* s.lat.
STE-U 4567/ CBS114274	*Passalora cajani*
STE-U 5250/ CBS111833	*Passalora cajani*
STE-U 5334/ CBS114275	*Passalora cajani*
STE-U 5335/ CBS113998	*Passalora cajani*
STE-U 5337	*Passalora cajani*
STE-U 5338	*Passalora cajani*
STE-U 5339/ CBS113999	*Passalora cajani*
STE-U 10456	*Passalora cajani*

CHAPTER 3

HISTOPATHOLOGY OF *UREDO CAJANI* IN PIGEONPEA VARIETIES

INTRODUCTION

Some of the most important diseases in agriculture are caused by rust fungi (Tani, 1988; Cummins and Hiratsuka, 2003). Particularly rusts of gramineous and leguminous crops are major factors in reducing agricultural productivity, over a broad geographical range. Rust fungi, in the order Uredinales, constitute one of the groups in the division Basidiomycota, in the class Urediniomycetes (Swann, *et al.*, 2001 in Cummins and Hiratsuka, 2003) and are distinguishable by their host specificity (Tani, 1988). Rust fungi parasitize fresh tissues of vigorously growing plants, unlike other kinds of plant pathogens which tend to attack weakened, poorly growing plants (Cummins and Hiratsuka, 2003). For this reason, intensive and extensive monoculture of economically important crops causes increasing problems with rust fungi. Rust pathogens are obligate parasites of living plants (Hiratsuka and Sato, 1982), and have co-existed and co-evolved with their hosts as components of a system much influenced by ecological conditions (Wahl *et al.*, 1984; Cummins and Hiratsuka, 2003).

Pigeonpea (*Cajanus cajan* (L.) Millsp.) rust, caused by *Uredo cajani* Syd., was first reported in Mpumalanga, South Africa in 2000 (Swart, Mathews and Saxena, 2000). High disease incidence observed on this new crop, suggested rust to be of importance in this particular area. Although economic damage by *U. cajani* is generally small or negligible in most countries, including Bermuda, India, Kenya and Venezuela (Nene, Sheila and Sharma, 1989 in Reddy, Sharma and Nene, 1990), it is significant in other areas, namely the Caribbean (Reed, 1987 in Anonymous). A sustainable disease management approach for the successful cultivation of pigeonpea in South Africa requires a sound understanding of abiotic and biotic factors that can affect this potentially important crop.

Characterization of the development of rust fungi in host plant tissues is important to establish an understanding of pathogenesis. Successful entry of rusts into a host requires development of special structures of the germ tube (Wolf, 1982), which are characteristic for each rust species. Pigeonpea rust histopathology has not previously been studied in detail. The purpose of this study was to investigate the infection process of *U. cajani* in six *C. cajan* varieties of

unknown resistance, and to identify possible resistance mechanisms active in the host plant.

MATERIALS AND METHODS

Plant varieties

Development of infection structures of *U. cajani* was studied in three medium-duration (MD) (ICP 6927, ICPL 87119 and ICPL 87051) and three long-duration (LD) (ICEAP 0053, ICEAP 0040, ICEAP 0020) *C. cajan* varieties. MD varieties having a maturity duration of 151 to 180 days, compared to LD varieties with a maturity duration of more than 180 days (Sharma *et al.*, 1981 in Reddy, 1990). Short-duration varieties were omitted from the study, because, when cultivated, sowing dates could be scheduled in such a manner that maturity and harvest would precede the date of disease onset.

Plant cultivation

Seeds of the varieties were obtained from the Lowveld Research Unit, Mpumalanga. Four seeds of each variety were planted in each of four replicate pots (300 ml) containing a steam-sterilized mixture of soil-peat moss (1:1 v/v). Plants were grown at 18-25 °C in a rust free, air-conditioned glasshouse cubicle. Once seedlings reached the V1-growth stage (the leaflets on the first [unifoliate] through to the last leaf node are unrolled), plants were thinned to two per pot and 50 ml of 10 g/L hydroponic nutrient solution (3:2:1 N:P:K) was added as a weekly soil drench to each pot.

Inoculation and incubation

Prior to inoculation of plants, the rust was multiplied on 2-month-old plants of variety ICPL 87119, which was observed to be highly susceptible in the field. Freshly harvested urediniospores of *U. cajani* were suspended in a light mineral oil (Soltrol 170®). A standardized spore concentration of 40 x 10^4 urediniospores/ml oil was prepared using a haemocytometer (Neubauer improved

bright-line, Marienfield, Germany) and light microscope (100x, Hund Wetzlar, Labotech, Germany). The adaxial as well as abaxial surfaces of 16 leaves of uniform age (on 4-month-old plants) per variety were marked (leaves were marked individually with a plastic tag around the stalk) and inoculated using compressed air. Microscopic examination of the spore suspension, sprayed onto water agar plates and incubated in the dark at 22 ˚C for 24 h, revealed that 62 % of the urediniospores germinated.

After inoculation, plants were dried at room temperature in fan-circulated air for 45 min, to prevent damage to oil-inoculated leaves, before placement in the dark in a dew-simulation chamber at 21-22 ˚C for 40 h. Thereafter plants were again dried for 1 h in fan-circulated air at room temperature before being transferred to a glasshouse cubicle, where conditions were maintained similar to those described for plant cultivation during the pre-inoculation period. The experiment was repeated in a second, independent study. The experiment was designed as a complete, randomized block design.

Post-inoculation sample preparation, staining and microscopic examination

Three leaves per variety were randomly sampled from different pots at 6, 12, 24, 48, 72, 96 hours post-inoculation (h.p.i.), and 7 and 14 days post-inoculation (d.p.i.). Leaf segments were cut from the middle part of inoculated leaves, 5 mm^2 for scanning electron microscopy and 10 mm^2 leaf sections for fluorescence microscopy.

Fluorescence microscopy. Samples were stained according to a modified Uvitex staining method (Rohringer *et al.*, 1977). Samples were cleared and fixed in ethanol:dichloromethane (3:1 v/v) + 0.15 % trichloroacetic acid for 24 h, washed 2x in 50 % ethanol for 15 min, twice for 30 min in 0.05 M sodium hydroxide (NaOH), and rinsed 3x with distilled water. Samples were then soaked in Tris/HCl buffer (pH 5.8) for 30 min, and stained for 5 min in 0.1 % Uvitex (Ciba-Geigy) (Niks & Dekens, 1991) in Tris/HCl buffer. Rinsing 4x with water preceded a final wash with 25 % aqueous glycerol (30 min). The stained leaf segments were stored in 50 % glycerol, containing trace lactophenol to prevent deterioration of the samples.

The leaf segments were mounted, abaxial side up, and used for microscopy (Rohringer *et al.*, 1977; Kuck *et al.*, 1981). Microscopic observations were carried

out at 20x magnification with a Nikon Labophot epifluorescence microscope using the filter combinations UV-1A (excitation filter 330 to 380 nm and barrier filter 420 nm) for fungal structures and B-2A (excitation filter 450 nm to 490 nm and barrier filter 520 nm) for observations of plant cell necrosis. Fungal structures fluoresced in a bright blue colour (Rohringer *et al.*, 1977), while unaffected host cells did not fluoresce. Host cells with a yellow-brownish fluorescence were considered necrotic. For 20 infection sites, for each of three replications, colony dimensions were measured with a calibrated eyepiece micrometer. Circular colony areas (mm^2) were calculated with the formula: $\pi \times r^2$. Colonies near leaf edges and coalescing colonies were excluded from all measurements.

Fungal development was quantified as the proportion of pre-haustorial and post-haustorial mother cell structures. Pre-haustorial mother cell structures consisted of germ tubes failing to produce appressoria (GT), germ tubes which formed appressoria but no further development occurred (AP) and non-penetrating appressoria (NPA), where appressoria did not penetrate the stomatal opening and further development of structures occurred on the leaf surface. Post-haustorial mother cell structures included early colonies (EC) and mature colonies, with (MC) or without sporulation (MCS). Early colonies can be defined as sites where six or less haustorial mother cells (HMC) were formed, whereas those with more than six were considered mature colonies (Niks, 1983). Colonies were also scanned for host cell necrosis. For these observations, 100 germinated spores (for each of three replications) were scanned per sampling period.

Scanning electron microscopy. Material was fixated for 24 h in 3 % glutaraldehyde, then washed twice with 0.05 M phosphate buffer (pH 6.8-7.2) and post-fixed in 1 % osmium tetroxide for 30 min. Samples were then washed with phosphate buffer before they were dehydrated in several ethanol steps. Dehydrated samples were critical-point dried in a Polaron dryer, sputter coated with gold in a Bio-Rad SEM coating system, and viewed with a JEOL WINSEM JSM-6400 scanning electron microscope operating at 5 kV. Fungal structures on the surface of the leaf segments were observed to confirm infection structures but not counted for quantitative analysis. The objective was to determine whether scanning electron microscopy (SEM) could be used to more accurately describe

the development of the rust infection structures.

Statistical analysis

An analysis of variance and the calculation of standard deviations were done using the statistical program NCSS 2000 (BMDP Statistical Software Inc., Los Angeles, CA). For each analysis, data from the first and second experiments were pooled if experiments, according to analysis of variance, were similar. Bartlett's test for homogeneity of variances (Bartlett, 1937) was conducted before data were pooled.

RESULTS

Infection process

Pigeonpea rust follows the most common infection process (Fig. 3.1) in all six host varieties. The urediniospore germinates to form a single, unbranched germ tube (Fig. 3.2 A & B). Each germ tube varied in length from being extremely short, with immediate appressorium formation (Fig. 3.3 A & B), to elongated as it grew among trichomes to the leaf surface (Fig. 3.3 C & D). When a germ tube comes into contact with the leaf surface, the tip swells to form an appressorium (Fig. 3.2 C & E). Appressoria were easy to distinguish morphologically, differing in shape and size, from small to large enough to cover the stoma completely. After the formation of an appressorium, an infection peg penetrates through the stomatal aperture (Fig. 3.4 A & B) and swells to form a substomatal vesicle (Fig. 3.2 D & F), from which one or more primary hyphae arise and elongate and upon contact with a mesophyll cell, differentiate into a thick-walled haustorial mother cell (Fig. 3.2 F). Infection occurred on the abaxial leaf surface, with occasional infection sites on the adaxial leaf surface.

Leaf morphology played a role in the growth pattern of the pathogen. Pigeonpea leaves are pubescent, more so on the lower than the upper leaf surface (Bisen & Sheldrake, 1981 in Reddy, 1990) with stomata scattered across the surface in an indistinguishable pattern. Germ tube growth was not only directed

by ridges (Fig. 3.3 E) and by position of simple (Fig. 3.5 A & B) and glandular trichomes (Fig. 3.3 F), but germ tubes also displayed elongated growth to grow through the trichome layer to reach the leaf surface. Germ tubes displayed two growth types, flattened against the leaf surface (Fig. 3.5 B & D), resembling germ tubes that collapsed after penetration, and the normal type (Fig. 3.3 D; Fig. 3.4 A).

Non-penetrating appressoria (Fig. 3.5 C & D) were noted in all six varieties, the appressorium forming over a stoma but not penetrating, with subsequent formation of further infection structures on the leaf surface. Penetration sometimes occurred after contact with another stoma (Fig. 3.5 D).

Pre-haustorial mother cell structures

Percentage germ tubes failing to produce appressoria (GT), appressoria failing to develop further (AP) and non-penetrating appressoria (where appressoria did not penetrate the stomatal opening and developed further on the leaf surface - NPA) are given in Table 3.1. For the sampling times, ranging from 12 h.p.i. to 96 h.p.i., there was little variation between the six varieties for each time period, except for a significantly higher number of germ tubes developing on variety ICEAP 0053 at 12 h.p.i. As the rust developed from 12 h.p.i., the number of germ tubes formed, decreased, whereas the number of appressoria increased during the time period of 12-96 h.p.i., then decreased again (Table 3.1). Formation of NPA followed the same pattern as that of AP formation, however a decrease in numbers was observed after 48 h.p.i.

At 7 d.p.i., the percentage pre-haustorial mother cell structures (PMCS) ranged from 77.7 % for ICPL 87051 to 95.7 % for IPC 6927 (Fig. 3.6 A). At 14 d.p.i., the percentages were visibly lower, varying from 63.8 % in ICEAP 0040 to 88.2 % in ICP 6927 (Fig. 3.6 A). In variety ICPL 87051, the percentage PMCS did not decrease significantly, which was also the case for the five other varieties.

Early colonies

Early colonies were observed from 12 h.p.i. in varieties ICPL 6927 and ICEAP 0040, and from 24 h.p.i. in all varieties (Table 3.1) but low percentages occurred throughout the sampling period. Percentage early colonies ranged from

1.8 % in ICEAP 0053 to 3.2 % in ICEAP 0040 and ICEAP 0020 at 7 d.p.i., and from 1.2 % in ICPL 87051 to 4.5 % in ICEAP 0040 (Fig. 3.6 B).

Colony formation

Mature colonies (where infection sites had more than six haustorium mother cells) were successfully established in all six varieties at 7 and 14 d.p.i. (Fig. 3.6 C). In all varieties, the percentage colony formation increased significantly ($P < 0.05$) with time, except for variety ICPL 87051. At 7 d.p.i. variety ICPL 87119 supported the lowest with1.5 % and ICPL 87501 highest colony formation at 19.7 %. This increases to 9.8 % in ICP 6927 and 31.7 % in ICEAP 0040 at 14 d.p.i. Percentage sporulating, mature colonies can be seen in Table 3.1. Sporulation was observed in two varieties at 7 d.p.i., ICPL 87051 and ICEAP 0053, however it was present in all varieties at 14 d.p.i., ranging from ICP 6927 with 4 % and ICEAP 0020 with 21.5 %.

Colony size

Measurements of colony areas at 14 d.p.i. showed variety ICP 6927 had the smallest (0.048 mm^2) and ICPL 87119 the largest (0.113 mm^2) colony area (Fig. 3.7). Host cell necrosis, associated with a hypersensitive reaction, did not occur, even at this advanced sampling time.

DISCUSSION

Many studies related to plant-rust interactions have been carried out with rust pathogens which typically penetrate through stomata (Tani, 1988). During the penetration phase, urediniospores germinate with germ tubes adhering onto hydrophobic plant surfaces (Tani, 1988). Germlings respond specifically to the plant surfaces in directional growth along surface ridges or lines on the leaf surface. These topographic factors aid the efficient orientation of germ tubes towards stomata (Heath, 1982). The germ tube stops growing upon reaching a stoma, and an appressorium forms. Many rust pathogens which invade their hosts

via stomata, subsequently develop a set of infection structures (Staples and Macko, 1984). After appressorium formation, an infection peg pushes through the stomatal opening, followed by differentiation of a substomatal vesicle in the stomatal cavity (Staples and Macko, 1984). Parasitism occurs when a haustorium is produced within an internal host cell (Staples and Macko, 1984).

Using fluorescence microscopy, infection structures of *U. cajani* on pigeonpea were easily distinguishable morphologically, even with the varying shapes of appressoria and different lengths of germ tubes. The infection process of *U. cajani* is similar to that of *Uromyces appendiculatus* (Pers.: Pers.) Unger (syn. *Uromyces phaseoli* var. *typica* (Pers.) G. Winter.) on bean (Wynn, 1976). Growth of both *U. cajani* and *Ur. appendiculatus* on the leaf surface is influenced by leaf topography, as demonstrated by Wynn (1976) for bean rust. This is in accordance with the findings of Allen *et al.* (1991), who determined that thigmotropism (capacity to recognise and respond to specific cues) plays a role in infection of certain rusts, and is specific for each rust species. Cuticular ridges on pigeonpea leaf surfaces direct *U. cajani* germ tube growth towards stomata. Growth is also directed by trichomes on the pubescent leaf surface (Bisen and Sheldrake, 1981 in Reddy, 1990) but trichomes do not seem to influence formation of infection structures in any way. Jacobs (1989a) found that the presence of epidermal hairs on wheat leaves also does not impede appressorium formation by *Puccinia recondita* f. sp. *tritici* Robb. ex Desm. (syn. *P. triticina* Erikss).

Infection by both *U. cajani* and *Ur. appendiculatus* (Wynn, 1976) occurs mainly on the abaxial leaf surface, through the stomatal openings and not by direct penetration of the host epidermis as does soybean rust, *Phakopsora pachyrhizi* H. syd. & P. Syd. (Bonde, Melching and Bromfield, 1976). By penetrating through the stoma, *U. cajani* applies a less confrontational, more complex strategy to gain access to plant tissues, characteristic of many biotrophic fungi (Dean, 1997).

The variety in growth habit of germ tubes might indicate a well adapted parasite. *Uredo cajani* can grow either short, flattened germ tubes or long germ tubes through and around trichomes in an attempt to locate a stoma. This pathogen has probably evolved this ability to overcome the morphological barrier that the trichomes, on the host's pubescent leaves presented.

Pigeonpea rust does not grow systemically inside the leaf, as is the case

with wheat yellow rust, *Puccinia striiformis* West. (Danial, 1994; Knott, 1989). With yellow rust, a single infection on a leaf can produce a long stripe containing many uredinia, which arise from systemic growth (Knott, 1989). A single infection point from *U. cajani* develops into a mature, sporulating colony (uredinium), from which newly formed urediniospores are spread to form new infection points.

To compare response differences between varieties used, the relative proportions of pre- and post-haustorial infection structures, observed at 7 and 14 d.p.i., are illustrated in Fig. 3.8., classified as germ tube (GT), appressorium formed (AP), non-penetrating appressorium (NPA), early colony (EC), successfully established colony without sporulation (C) and sporulating colony (CS). According to these data, colony establishment occurred at a higher percentage in ICPL 87051 at 7 d.p.i. than in any other variety, suggesting it to be highly susceptible at this sampling time. Variety ICPL 87119 seemed to be the least susceptible, however at 14 d.p.i., ICP 6927 displayed least susceptibility and ICEAP 0020 had the highest establishment of colonies. The general observation was that all varieties responded similarly, with a high percentage of pre-haustorial mother cell structures (PMCS) at 7 d.p.i., with more colonies being formed and starting to sporulate by 14 d.p.i. Niks (1981; 1982) and Jacobs (1989a) found that spore germination, appressorium formation and stomatal penetration are not affected by resistance of partially resistant wheat. The lack of significant differences in the number of PMCS between varieties in this present study, suggests that the varieties studied all have partial susceptibility.

Jacobs (1989b) reported genotypic effects of wheat leaf rust to only be expressed after the formation of the first haustorial mother cells, which suggests a certain point where differences between resistance of varieties can be observed. The process results in retardation or restriction of fungal growth, which in turn leads to production of fewer, otherwise normal pustules or a slower rate of pustule appearance (slow-rusting). The latter phenomenon may, therefore, be viewed a defence mechanism (Heath, 1982) and a component of partial resistance (Parlevliet, 1978 in Niks, 1983). Jacobs (1989c) determined that the number of *Pu. recondita* f. sp. *tritici* colonies not sporulating at time of observation in partially resistant wheat genotypes (*Triticum aestivum* L.), should be considered indicative of the growth delaying capacity of partial resistance. *Uredo cajani* colonies only

started sporulating on most varieties at 14 d.p.i., showing the characteristic slow rust rate.

Low and equal percentages of early colony formation were observed throughout the sampling time. This is probably not indicative of early abortion, as described in gramineous crops (Niks, 1983), but rather a slow colony establishment that starts soon after inoculation and gradually culminates in sporulating colonies. The phenomenon does not play a significant role in slowing the rate of colony formation or reducing the number of colonies, similar to what happens in wheat leaves of partially resistant wheat genotypes (Jacobs, 1989c). Similarly, low and equal percentages of aborted infection structures were observed throughout the sampling time, suggesting that this reaction does not play a significant role in resistance either. Variety ICP 6927 was the one exception and showed least susceptibility at 14 d.p.i., with the percentage aborted appressoria significantly higher than in the other five varieties. Where aborted penetration was observed, it could be argued that a stomatal trigger was lacking, and further growth suggests that this is a morphological resistance mechanism of some sort. Aborted penetration is associated with non-host reactions occurring early in the infection process (Heath, 1981; 1982), although in pigeonpea this does not seem to be the case.

Typically, penetration of a host plant by a rust pathogen is accompanied by morphological host responses, such as collapse of host tissue, usually only seen after formation of the first haustorium is initiated (Heath, 1982). The most common response of incompatible hosts after the first haustorium develops, is necrosis of the haustorium-containing cells (Heath, 1982). The necrotic area can be seen under ultraviolet light, where it may show autofluorescence, probably in association with phenol accumulation (Rohringer and Heitefuss, 1984). The autofluorescencing necrotic cells can be readily distinguished from the weak autofluorescence exhibited by normal cells (Rohringer and Heitefuss, 1984). Necrosis of the invaded cells, in some host-parasite interactions, may be very soon after the first haustorium is initiated (Littlefield and Aronson, 1969; Heath, 1971). In other interactions, however, it may not begin until several days after inoculation, by which time several, apparently normal haustoria may have been formed (Heath, 1972; Mares 1979; Rohringer, Kim and Samborski, 1979). Host response is

however not seen in all interactions, the hosts either being insensitive to fungal substances, or their responses do not result in a change in cell appearance (Heath, 1974; Heath, 1982). Necrosis, usually observed in association with infection (Elliston, Kuć and Williams, 1971; Heath, 1980; Carroll, 1991) was not observed around the *U. cajani* infection point, even around colonies at 14 d.p.i.

When combining the proportions of infection structures with colony area, it is clearly evident that ICP 6927 is least and ICPL 87119 most susceptible to rust, even with a significant difference in percentage pre- and post-haustorial mother cell structures between these two varieties. Susceptibility levels in medium-duration varieties were variable, which might make selection of varieties difficult within this duration type. Long-duration varieties displayed a more consistent level of susceptibility, possibly giving a better initial idea of the disease severity that can be expected.

The present study indicates that susceptibility of a non-hypersensitive nature, identified by slow-rusting, is present in pigeonpea varieties. This mechanism seems to be sufficient to restrict *U. cajani* infection, and probably explains why pigeonpea rust is considered a disease of lesser importance. Susceptibility was present in all varieties, to varying degrees. Uredinium size and time of colony formation can be used to determine differences in susceptibility between varieties.

With the infection process known, further studies can now be done to explore other sub-components of the disease triangle, which influence pigeonpea rust. Possible resistance and latent period can be investigated, and differential host susceptibility levels can be applied to measure disease severity in field. Studies with *U. cajani* could include non-hosts to provide a better understanding of possible exclusion mechanisms in the pre-haustorial mother cell structure phase.

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Figure 3.1. Diagram of infection structures produced by the pigeonpea rust fungus, *Uredo cajani* (adapted from Staples and Huang, 1982 in Staples and Macko, 1984). Urediniospore (US), germ tube (GT), appressorium (AP), infection peg (IP), substomatal vesicle (SSV), infection hyphae (IH) and haustorial mother cell (HMC), trichome (TC), stoma (ST).

Figure 3.2. *Uredo cajani* infection structures, stained with Uvitex and viewed with a fluorescence microscope. **A:** Urediniospore (US). **B:** US with emerging germ tube (GT). **C & E:** GT and appressorium (AP). **D:** AP over stomatal aperture (SA) and vesicle (SSV) forming in stomatal cavity. **F:** SSV with primary infection hyphae (IH) and haustorial mother cells (HMC).

Figure 3.3. Germ tubes (GT) (of varying lengths) and appressoria (AP) of *Uredo cajani*, viewed with a fluorescence microscope (**A & C**) and a scanning electron microscope (**B & D**). Urediniospore (US), singular trichome (ST). **E:** GT growth directed by ridges on the leaf surface. **F:** GT exhibiting flattened growth around a glandular trichome (GL), viewed with a scanning electron microscope.

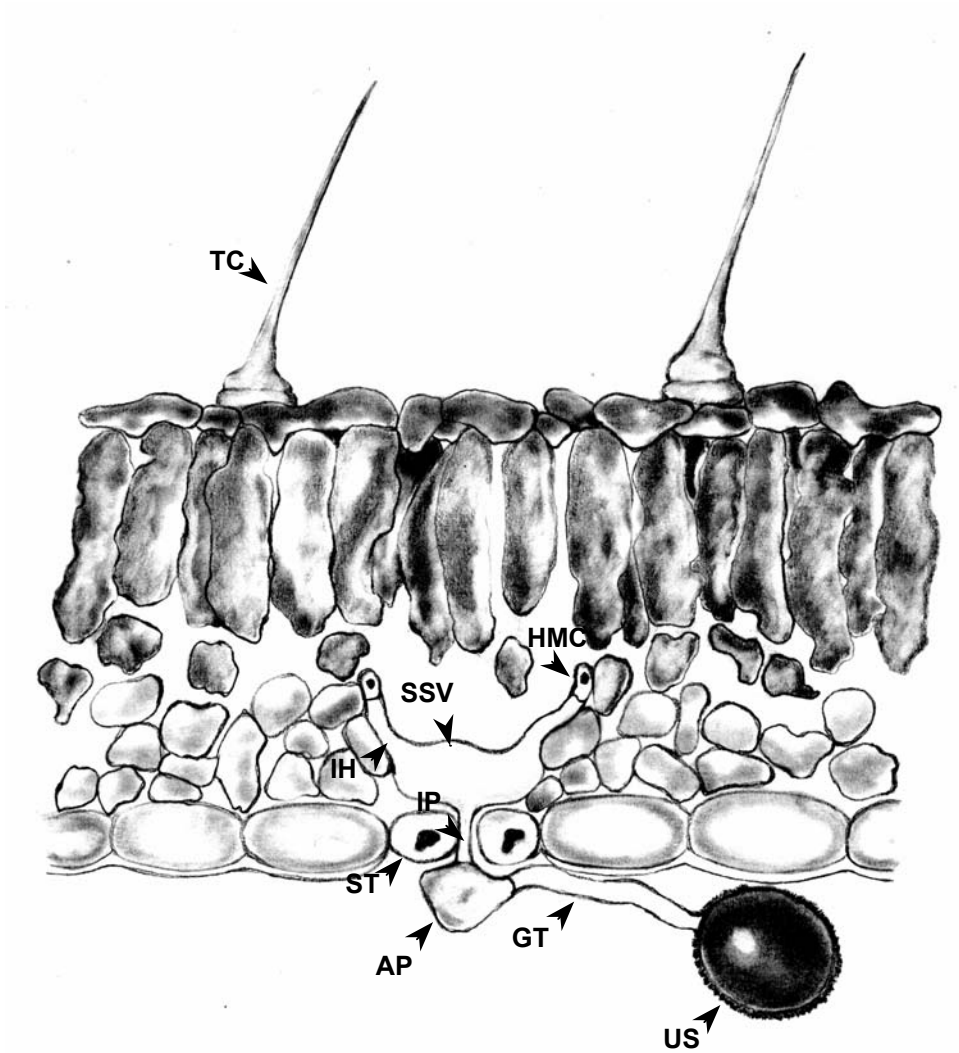
Figure 3.4. A & B: Germinated *Uredo cajani* urediniospore (US) with appressorium (AP) formation and penetration of the pigeonpea leaf through the stomatal aperture (SA). Germ tube (GT).

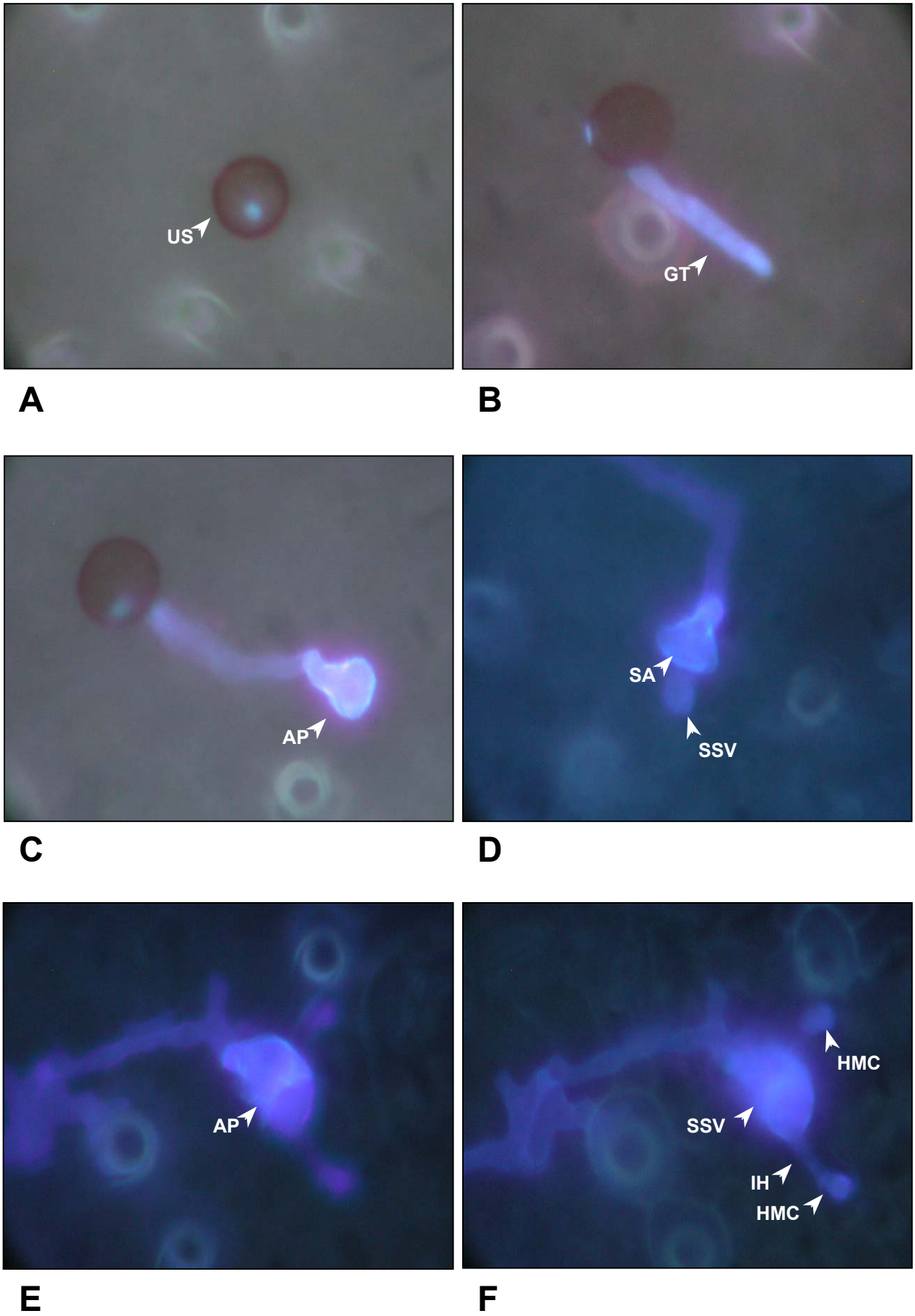
Figure 3.5. *Uredo cajani* germ tubes (GT) directed by trichomes and displaying flattened growth habit, viewed with a fluorescence microscope (**A & C**) and a scanning electron microscope **(B & D**). **A & B:** Growth of the GT emerging from the urediniospore (US) is influenced by the singular trichome (ST). **C & D:** Further growth occurs from a non-penetrating appressorium (NPA). The GT continues to grow over another stoma, around a ST and finally forms another appressorium (AP) over a third stoma.

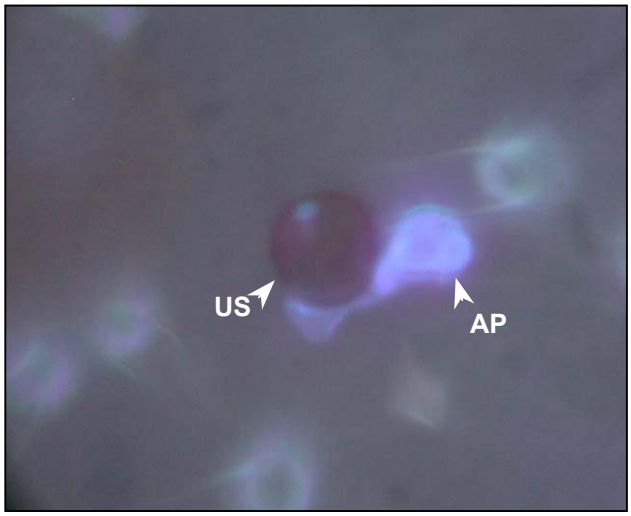
Figure 3.6. Percentage pre-haustorial mother cell structures (**A**), early colonies formed (**B**) and colony formation (**C**) of *Uredo cajani* determined 7 and 14 days post-inoculation, on the abaxial leaf surface of six varieties of *Cajanus cajan*. Standard deviation is represented by error bars.

Figure 3.7. Colony area (mm^2) of *Uredo cajani* measured 14 days post-inoculation in six varieties of *Cajanus cajan*. Standard deviation represented by error bars.

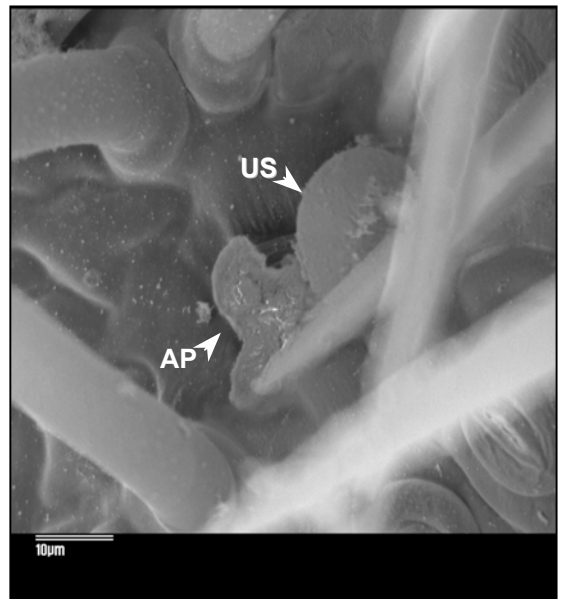
Figure 3.8. Percentage of infection sites of *Uredo cajani* classified as germ tubes (GT), appressoria formed (AP), non-penetrating appressoria (NPA), early colonies (EC), successfully established colonies without sporulation (C) and sporulating colonies (CS). Six *Cajanus cajan* varieties were used and determinations were made at 7 and 14 days post-inoculation on the abaxial leaf surfaces.


TC
HMC
SSV
IH
IP
ST
AP
GT
US

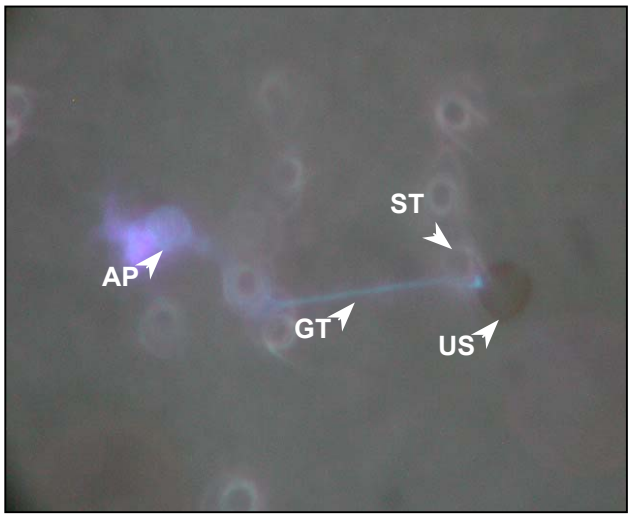

US
GT
A
B
AP
SA
SSV
C
D
AP
HMC
SSV
IH
HMC
E
F


US
AP

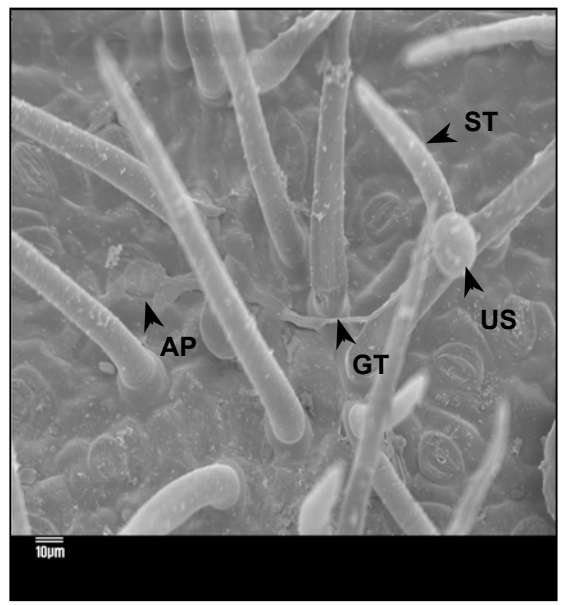
A


US
AP
10µm

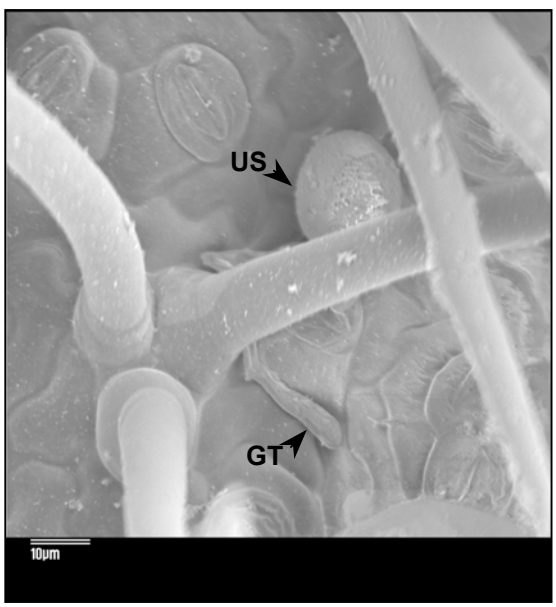
B


ST
AP
GT
US

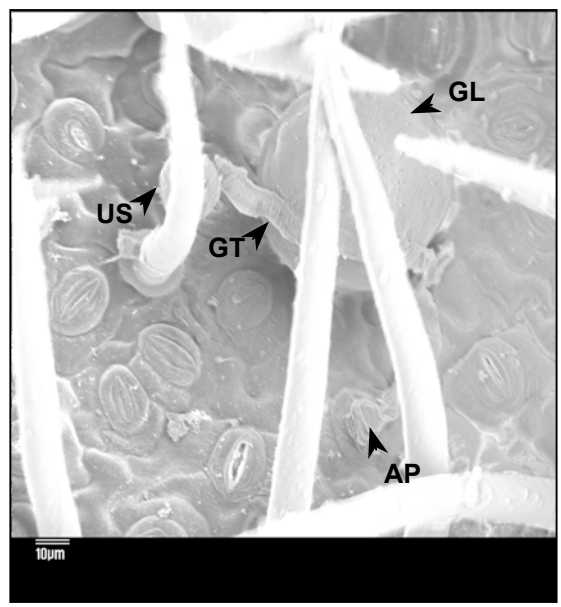
C


ST
US
AP
GT
10µm

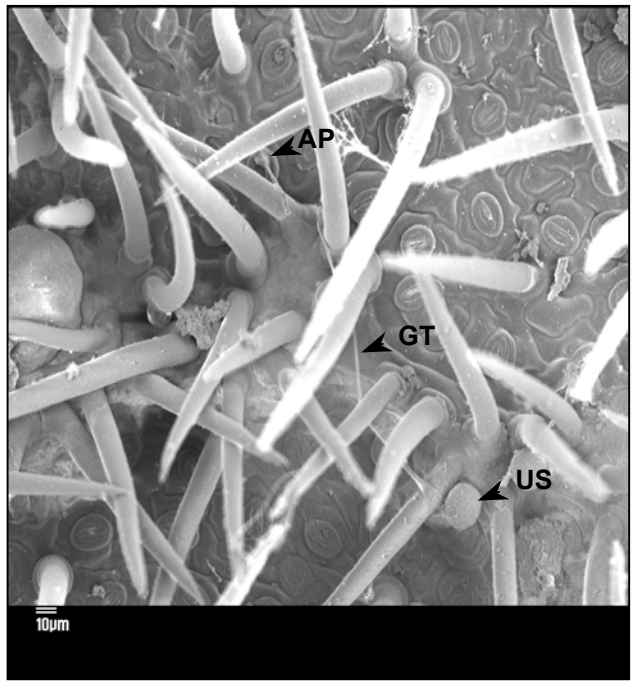
D


US
GT
10µm

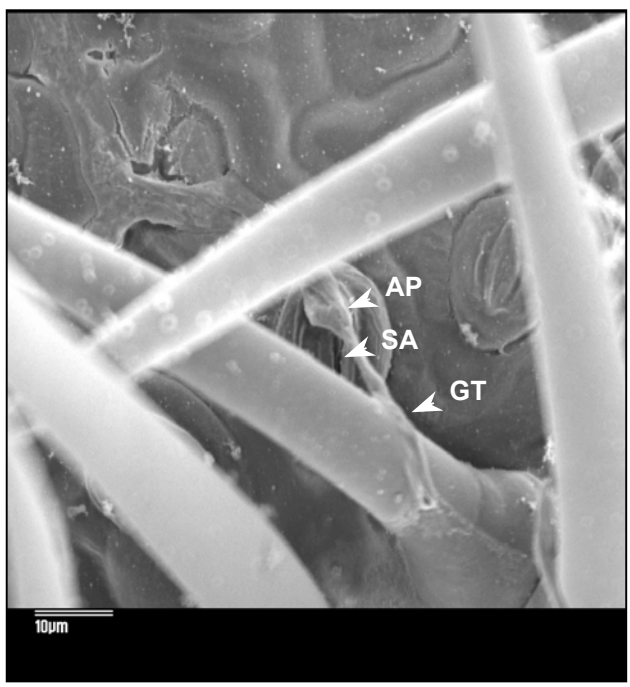
E


GL
US
GT
AP
10µm

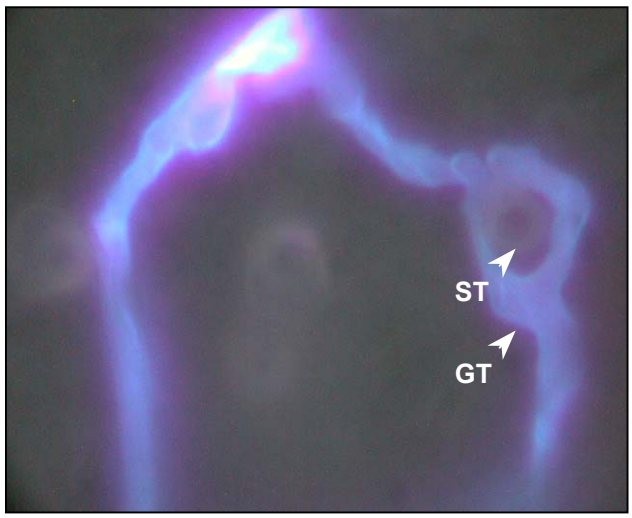
F



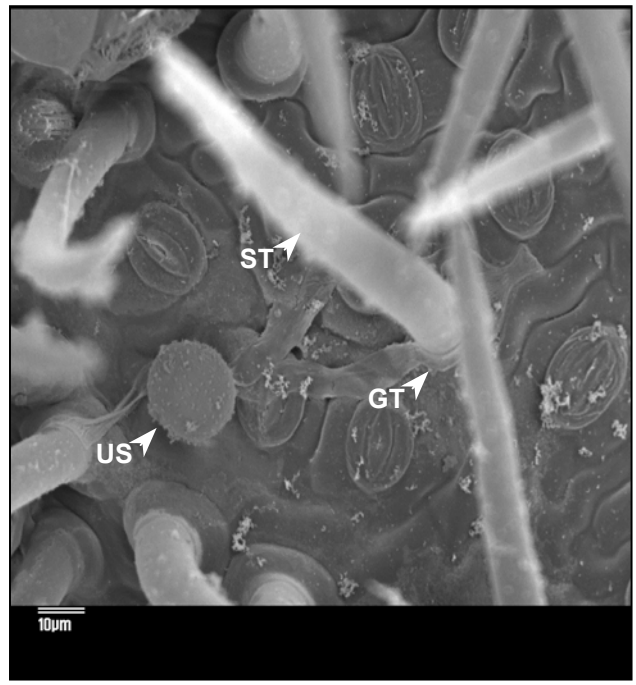
A



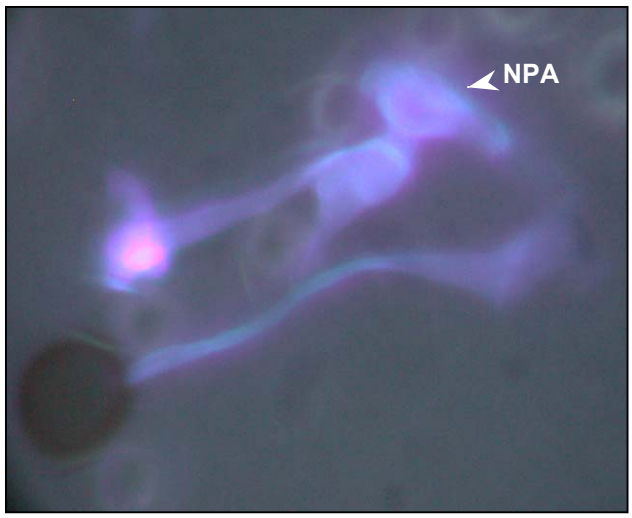
B



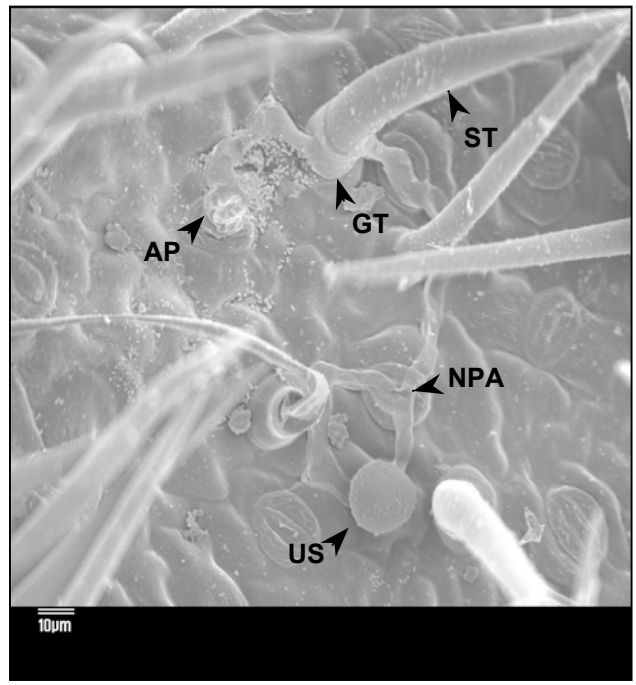
A



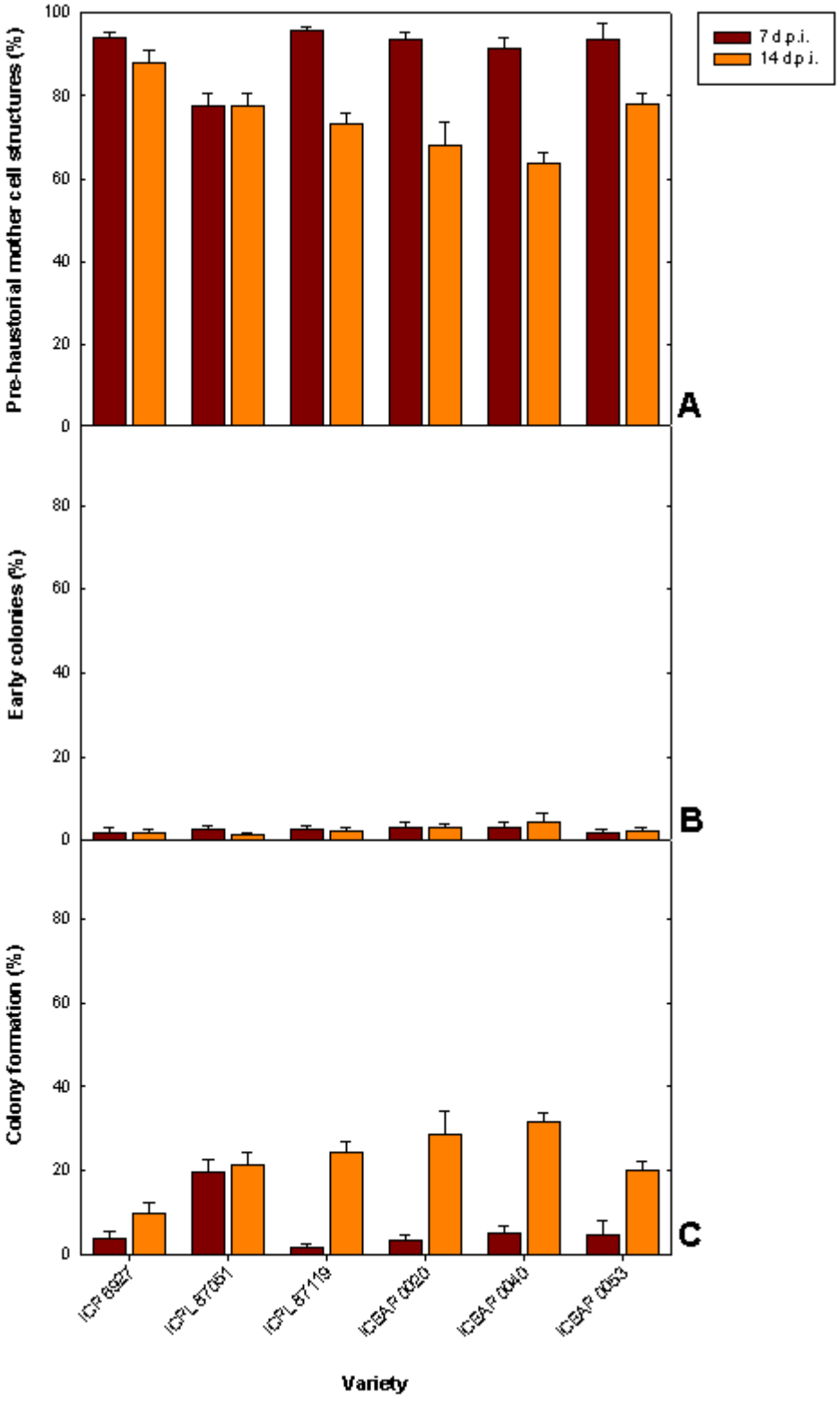
B

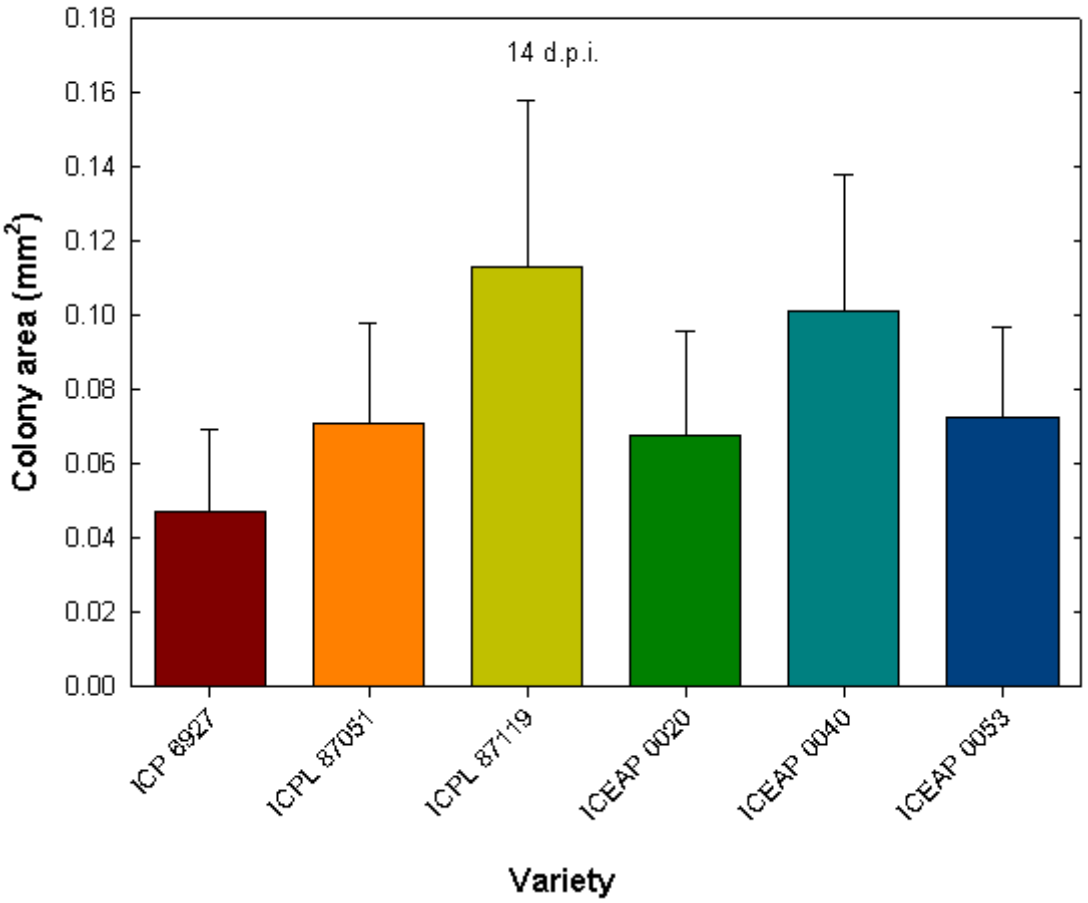


C



D


7 d p.i.
14 d p.i.
Pre-haustorial mother cell structures (%)
Early colonies (%)
Colony formation (%)
A
B
C
0
20
40
60
80
100
ICP 6927
ICPL 87051
ICPL 87119
ICEAP 0020
ICEAP 0040
ICEAP 0053
Variety


14 d.p.i.
Colony area (mm2)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
ICP 8927
ICPL 87051
ICPL 87119
ICEAP 0020
ICEAP 0040
ICEAP 0053
Variety

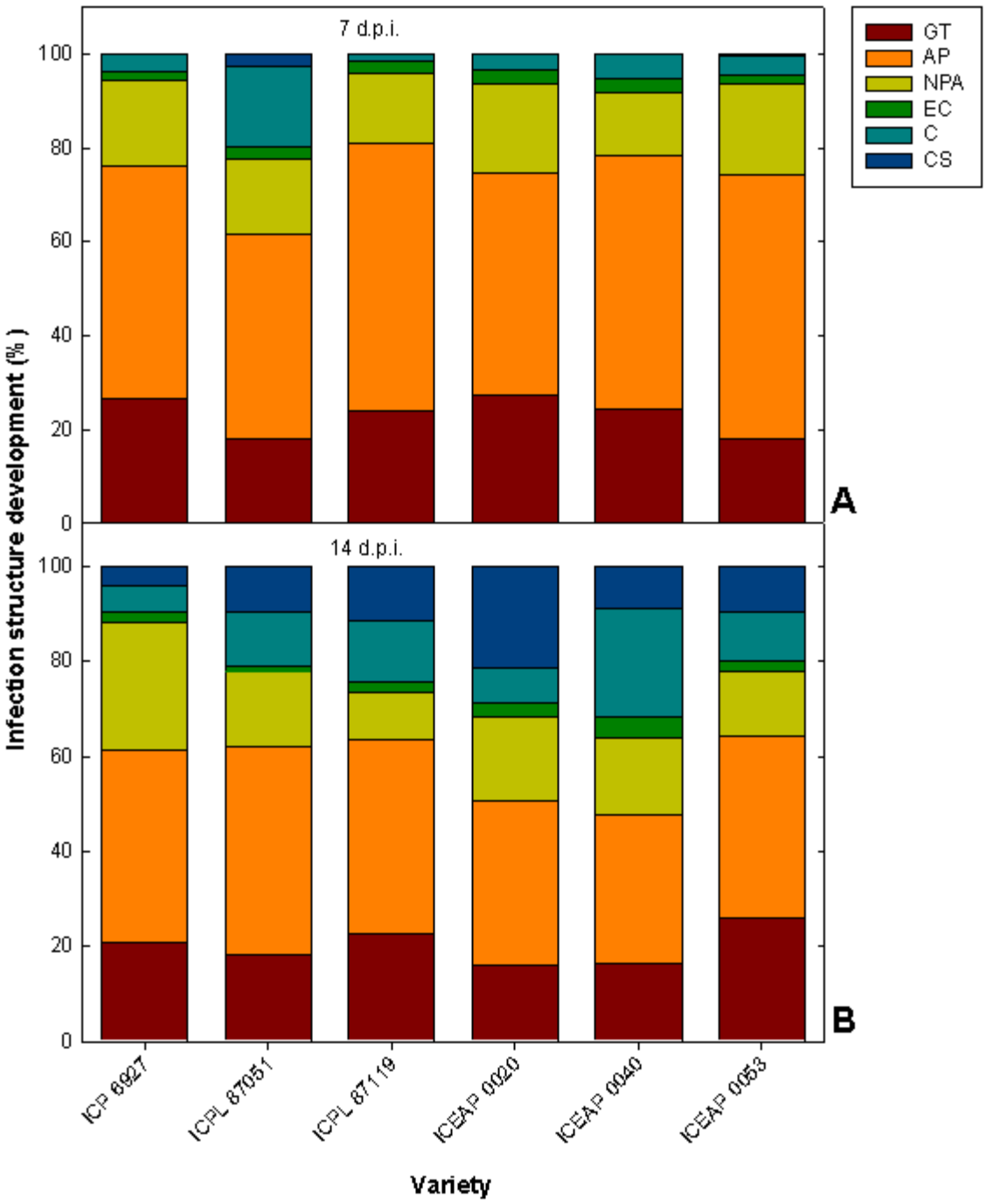

7 d.p.i.
14 d.p.i.
GT
AP
NPA
EC
C
CS
Infection structure development (%)
0
20
40
60
80
100
A
B
ICP 6927
ICPL 87051
ICPL 87119
ICEAP 0020
ICEAP 0040
ICEAP 0053
Variety

Table 3.1 Infection structures[a] formed by *Uredo cajani* in *Cajanus cajan* varieties as determined by fluorescence microscopy for six post-inoculation sampling times

		Varieties					
		Medium-duration type			Long-duration type		
Infection structure	Sampling time	ICPL 6927	ICPL 87051	ICPL 87119	ICEAP 0020	ICEAP 0040	ICEAP 0053
PRE-HAUSTORIAL MOTHER CELL STRUCTURES							
Precolonization (%)							
No appressorium formed	12 h.p.i.	53.5 ± 1.6	44.2 ± 3.8	46.2 ± 2.5	46.7 ± 4.3	51.0 ± 3.6	67.5 ± 5.7
	24 h.p.i.	42.2 ± 9.7	20.7 ± 4.5	40.5 ± 4.2	28.8 ± 7.8	32.0 ± 3.2	29.2 ± 4.3
	48 h.p.i.	18.2 ± 2.8	23.7 ± 3.9	37.7 ± 2.9	24.7 ± 4.7	23.0 ± 3.6	26.5 ± 1.9
	96 h.p.i.	33.7 ± 5.9	24.7 ± 4.1	33.8 ± 5.6	21.0 ± 1.7	28.2 ± 4.8	24.3 ± 3.6
	7 d.p.i.	26.7 ± 2.5	18.3 ± 2.3	24.3 ± 3.7	27.7 ± 5.8	24.7 ± 2.7	18.3 ± 4.7
	14 d.p.i.	21.0 ± 2.6	18.3 ± 3.3	22.7 ± 3.4	16.2 ± 2.5	16.5 ± 3.5	26.0 ± 3.8
Appressorium formed without further development	12 h.p.i.	42.2 ± 3.5	47.5 ± 3.3	48.2 ± 3.1	44.5 ± 4.5	46.0 ± 3.6	27.5 ± 6.1
	24 h.p.i.	44.3 ± 6.3	56.3 ± 5.0	44.5 ± 3.5	46.0 ± 6.9	46.3 ± 4.0	53.8 ± 5.6
	48 h.p.i.	49.5 ± 6.8	53.0 ± 4.6	48.2 ± 2.6	51.0 ± 2.3	49.8 ± 6.5	48.5 ± 5.3
	96 h.p.i.	43.7 ± 6.5	55.3 ± 4.8	50.5 ± 5.1	56.0 ± 3.0	55.2 ± 4.0	42.2 ± 4.8
	7 d.p.i.	49.3 ± 1.9	43.3 ± 3.3	56.5 ± 4.1	46.8 ± 4.1	53.8 ± 4.0	55.8 ± 3.31
	14 d.p.i.	40.3 ± 2.9	43.7 ± 6.9	40.7 ± 1.9	34.3 ± 4.0	31.3 ± 4.5	38.2 ± 5.4
Abortive penetration (%)							
Non-penetrating appressorium	12 h.p.i.	3.8 ± 2.2	8.3 ± 2.7	5.7 ± 2.0	8.8 ± 2.3	2.8 ± 1.2	5.0 ± 1.1
	24 h.p.i.	11.8 ± 3.0	20.5 ± 3.7	8.0 ± 1.4	23.5 ± 2.6	19.7 ± 5.3	15.3 ± 3.8
	48 h.p.i.	29.7 ± 8.2	18.8 ± 5.3	10.5 ± 1.9	22.3 ± 2.6	24.5 ± 3.6	21.8 ± 6.2
	96 h.p.i.	19.2 ± 5.6	15.7 ± 2.42	10.5 ± 5.3	18.2 ± 3.5	13.2 ± 3.3	30.0 ± 4.6
	7 d.p.i.	18.2 ± 3.2	16.0 ± 1.9	14.8 ± 2.6	19.0 ± 3.9	13.2 ± 2.4	19.3 ± 3.2
	14 d.p.i.	26.8 ± 3.7	15.7 ± 2.0	10.0 ± 2.0	17.7 ± 2.5	16.0 ± 3.7	13.7 ± 2.6

Table 3.1 Infection structures formed by *Uredo cajani* in *Cajanus cajan* varieties as determined by fluorescence microscopy for six post-inoculation sampling times

		Varieties					
		Medium-duration type			Long-duration type		
Infection structure	Sampling time	ICPL 6927	ICPL 87051	ICPL 87119	ICEAP 0020	ICEAP 0040	ICEAP 0053
POST-HAUSTORIAL MOTHER CELL STRUCTURES							
Early colony formation (%)							
Early colonies (<6 HMC)	12 h.p.i.	0.5 ± 0.6	0	0	0	0.2 ± 0.4	0
	24 h.p.i.	1.7 ± 1.4	2.5 ± 1.4	7.0 ± 1.6	1.7 ± 0.8	2.0 ± 0.6	1.7 ± 0.8
	48 h.p.i.	2.7 ± 1.2	3.83 ± 1.2	3.7 ± 1.2	2.0 ± 1.3	2.7 ± 0.5	3.2 ± 1.5
	96 h.p.i.	3.5 ± 0.6	4.3 ± 0.8	5.2 ± 1.5	4.8 ± 0.8	3.5 ± 1.4	3.5 ± 1.8
	7 d.p.i.	2.0 ± 0.9	2.7 ± 0.8	2.8 ± 0.8	3.2 ± 1.2	3.2 ± 1.3	1.8 ± 1
	14 d.p.i.	2.0 ± 0.6	1.2 ± 0.8	2.3 ± 0.8	3.2 ± 1.9	4.5 ± 1.9	2.2 ± 0.8
Colony formation (%)							
Non- sporulating colonies	12 h.p.i.	0	0	0	0	0	0
	24 h.p.i.	0	0	0	0	0	0
	48 h.p.i.	0	0	0	0	0	0
	96 h.p.i.	0	0	0	0	0	0
	7 d.p.i.	3.8 ± 1.5	16.8 ± 2.5	1.5 ± 0.8	3.3 ± 1.2	5.2 ± 1.5	4.3 ± 2.6
	14 d.p.i.	5.8 ± 1.5	11.3 ± 3.4	12.7 ± 2.5	7.2 ± 1.5	22.8 ± 2.3	10.5 ± 3.2
Sporulating colonies	12 h.p.i.	0	0	0	0	0	0
	24 h.p.i.	0	0	0	0	0	0
	48 h.p.i.	0	0	0	0	0	0
	96 h.p.i.	0	0	0	0	0	0
	7 d.p.i.	0	2.8 ± 0.8	0	0	0	0.3 ± 0.8
	14 d.p.i.	4.0 ± 1.1	9.8 ± 2.1	11.7 ± 2.6	21.5 ± 4.8	8.8 ± 3.1	9.5 ± 3.2
Uredium area (mm^2)	14 d.p.i.	0.048 ± 0.022	0.071 ± 0.027	0.113 ± 0.045	0.068 ± 0.028	0.102 ± 0.037	0.073 ± 0.025

[a] Data presented as means and standard deviations.

CHAPTER 4

DISEASE ASSESSMENT AND YIELD LOSS IN PIGEONPEA INFECTED WITH *UREDO CAJANI*

INTRODUCTION

Pigeonpea (*Cajanus cajan* (L.) Millsp.) is a versatile legume widely cultivated in the tropics and subtropics (Silim, 2001). The plant has been used for centuries in intercropping systems, and is an ideal source for food, animal feed and firewood (Nene and Sheila, 1990). Although there is a huge demand for its seeds (dhal) by the Asian community in South Africa, pigeonpea is still considered a new crop in this country, with production increasing in Eastern and Southern Africa (Mathews, 2000). Successful commercial cultivation of pigeonpea depends on a sustainable disease management approach.

Pigeonpea rust, caused by *Uredo cajani* Syd., was first reported in South Africa in Mpumalanga, in 2000 (Swart, Mathews and Saxena, 2000). *Uredo cajani* attacks the leaves and is visible as dark brown pustules full of urediniospores on the abaxial leaf surfaces (Fig. 4.1 A). Severe rust infection can cause extensive leaf drop, which can reduce the yield and quality of this crop (Reddy *et al.*, 1993). Economic losses due to rust are however small or negligible in most areas (Nene, Sheila and Sharma, 1989 in Reddy, Sharma and Nene, 1990), with the exception of the Caribbean (Duke, 1983; Reed, 1987 in Anonymous). A high incidence of rust observed in Mpumalanga (Fig. 4.1 B) in 2001, suggested this disease to be of some importance in South Africa.

Control measures aimed at controlling pigeonpea rust include the use of fungicides and resistant cultivars (Reddy *et al.*, 1993). Comparative studies of yields of different maturation varieties have been done (Smith, Baudoin and Mergeai, 2001), as well as studies on environmental influences on pigeonpea and the more important diseases of the crop (Singh and Chauhan, 1992; Odeny, 2001).

Systematic screening methods, being developed and adapted over time, have proved useful to evaluate leaf diseases of crops, including rust and leaf spot on groundnut (Subrahmanyam *et al.*, 1995), anthracnose of sorghum (Sinha, 1992) and gray leaf spot in maize (Ward, Laing and Rijkenberg, 1997). Screening methods are mostly based on growth stage and/or percentage leaf area affected, and certain specific requirements are often set for each rating system. For instance, when screening groundnut for rust or leaf spot, all leaves on the main

stem should be examined (Subrahmanyam *et al.*, 1995). Pigeonpea varieties have different growth habits, ranging from compact/erect with acutely angled branches (< 30˚), to spreading types with branch angles as large as 60˚ (Whiteman, Byth, and Wallis, 1985). Some pigeonpea plants of medium-duration and long-duration varieties grow over two metres in height. A quick, effective rating scale to screen the whole plant is important, in order to prevent misleading disease ratings. Few references to rust screening methods on pigeonpea could however be found and none of these take growth habit into consideration. Echavez-Badel and Bosques-Vega (1998) screened pigeonpea genotypes in field trials using a rating scale based on disease reaction (susceptible, intermediate or resistant) and pustule size.

The main objective of this study was to develop a disease rating system for the pigeonpea-rust pathosystem, which can be used to determine differences between varieties being tested for cultivation in South Africa. A secondary objective was to determine yield and quality loss due to rust and the efficacy of selected fungicides.

MATERIALS AND METHODS

Varieties

Six pigeonpea varieties, namely three medium-duration (MD) (ICP 6927, ICPL 87051, ICPL 87119) and three long-duration (LD) (ICEAP 0020, ICEAP 0040, ICEAP 0053) types were evaluated. Short-duration varieties were omitted from the study, since maturity and harvest would precede the date of disease onset at the chosen site. The varieties were planted at the Lowveld Research Unit site near Nelspruit in Mpumalanga in 2001. Three row plots 4.9 m long, spaced 90 cm apart, containing 10 plants each, were planted for each variety, according to a split plot design with four replications. No inoculations with *U. cajani* were conducted.

Spraying programme

Treatments consisted of two fungicides sprayed alternately, every two weeks and a control treatment, which was not sprayed at all. Application was made using a knapsack sprayer calibrated to apply the recommended rates. The fungicides Punch-Xtra® (flusilazole/carbendazim - 125 g +250 g/L) and Amistar® (azoxystrobin - 500 g/L) were applied individually starting with Punch-Xtra at 55 days after planting (DAP) on all plots, except the control treatments. Punch-Xtra® was sprayed to control various leaf spots and rust, and Amistar® to control various leaf spots and possibly rust, respectively.

Disease and yield assessment

Two disease assessments were made, one in May and one in June 2002, using a specially developed rating scale (Fig. 4.2 and Table 4.1). The scale was adapted for pigeonpea from an evaluation scale for rust on groundnut (Subrahmanyam *et al.*, 1995). The modified scale is based on the distribution of the rust through the whole plant, as well as extent of leaf area damaged, taking into account the growth habit of the plant. The rating scale, 0 (no disease) to 3 D (100 % diseased) was numbered from one to 13 for purpose of analysis. For each seed sample, the 100-pod seed weight was determined.

Protein analysis

An analysis of total crude protein was done using the Leco FP-528 Protein/ Nitrogen Determinator (Leco® Corporation, U.S.A.) to determine effect of rust on protein content of seeds.

Data analysis

Data analysis (ANOVA) for the disease assessment was done using NCSS 2000 (BMDP Statistical Software Inc., Los Angeles, California, U.S.A.). Data analysis of yield was done using SAS (SAS System, SAS Institute Inc., North Carolina, U.S.A.).

RESULTS AND DISCUSSION

The rating system developed in the current study had to meet with specific requirements for the screening of pigeonpea varieties. Firstly, it took into account the spread of the pathogen through the plant from the oldest leaves upwards, as well as measuring the spread on individual branches. Secondly, the whole plant was rated, instead of certain parts, resulting in more accurate disease ratings. Most importantly, and novel to a rating system, *C. cajan* varieties exhibit different growth habits (Fig. 4.2), which the rating scale had to accommodate as well. The rating system developed here met with these requirements and provided a simple and effective guide to determine varietal differences based on disease incidence, which can be applied when screening new varieties for resistance.

Disease ratings for the Nelspruit field trial are given in Fig. 4.3. In sprayed plots, no disease symptoms were recorded during the two assessments using the rating scale. In unsprayed plots, disease severity increased significantly ($P < 0.05$) from the first assessment in April to the second in May of the same year. Disease ratings also differed significantly ($P < 0.05$) between varieties. The ratings varied between 3 and 5.7 on ICPL 87119 and ICP 6927 respectively in April, and 9.2 and 11.9 on ICEAP 0020 and ICP 6927 respectively in May, 2002. The higher disease incidence observed in MD varieties which have a shorter maturity duration (151 to 180 days) than the LD varieties (>180 days) (Sharma *et al.*, 1981 in Reddy, 1990), was probably due to disease severity increasing with the onset of flowering (Reddy *et al.*, 1993). All varieties used in this trial were susceptible to the pathogen, with variety ICP 6927 most susceptible and ICEAP 0020, least susceptible. Disease severity on individual plants nearly doubled between the two assessment dates, affecting significantly ($P < 0.05$) more of the leaf area.

Preliminary analysis of total crude protein showed no significant ($P < 0.05$) difference in protein content between the treated (17.5 %) and untreated (18.2 %), diseased plots and thus protein content was not further investigated. This is consistent with the findings of Haigh and Bradshaw (1998), who showed that fungicide treatment produced no significant effect on nutritive value of either winter or spring oats. Wang *et al.* (2002) determined that a range of fungicide treatments

tested on wheat against leaf spotting diseases did not generally affect yield or grain protein concentration, and these effects were relatively consistent among genotypes. They also concluded that fungicide effect, on yield and protein concentration, was dependant on host age as well as environment, rather than the effect of disease (Rees and Syme, 1981). The effect of fungicide treatments on protein concentration, in trials done by Ruske, Gooding and Jones (2003) on wheat, varied between cultivars but in the few instances, where small reductions in protein concentration were observed, it could be compensated for by the application of extra nitrogen.

Based on 100-pod seed weight, LD varieties produced higher yields when means were pooled (Fig. 4.4). Significant interactions ($P < 0.05$) were observed between yield and varieties, whereas no interactions existed between treatments, yield and varieties. For treated and untreated plots the highest yielding variety was ICP 6927 and the lowest yielding was ICPL 87119. Surprisingly, all varieties, with the exception of ICEAP 0040 and ICEAP 0053, yielded more in unsprayed plots than in sprayed plots. Fungicide treatments therefore did not increase seed yield significantly ($P < 0.05$). This is contrary to what was expected and in contrast to results of fungicide trials against pea rust (*Uromyces fabae* (Grev.) Fuckel), where fungicide treatment increased yield (Singh and Singh, 1997). Haigh and Bradshaw (1998) also found that fungicide treatments produced a significant increase in grain yield in winter oats, as did Ruske *et al.*(2003) with strobilurin fungicide treatments in a range of wheat cultivars.

The reduction in yield observed in the present study could possibly be due to inhibiting effects of fungicides on the host plant and/or on *Rhizobium* spp. associated with *C. cajan*, resulting from run-off. Davis *et al*. (1985) in Kuck, Scheinpflug and Pontzen (1995) reported that flusilazole caused no phytotoxic effects on tree fruits at prescribed concentrations. Thomas (1974) in Delp (1995) also found that carbendazim, at recommended concentrations, was not phytotoxic, but rather displayed a yield boosting ability due to cytokinin-like properties. However, Onim (1990) in Kimani (2001) reported depressed grain yield in pigeonpea, when evaluating carbendazim (50 %) for control against Passalora (Mycovellosiella) leaf spot, and attributed this reduction to possible phytotoxicity. Odeyemi and Alexander (1977) showed that fungicides used as seed treatments,

can influence *Rhizobium* spp. negatively as well as positively, depending on active ingredients. Kutcher *et al.* (2002) observed no effects. These findings need to be carefully considered since choice of fungicide can simultaneously allow for seed protection without affecting N_2-fixation (Odeyemi and Alexander, 1977). This being said, it is recommended that specific experiments should be conducted to investigate fungicide influence on *Rhizobium* spp. of pigeonpea.

Based on data of the present study, a significant ($R^2 = 0.975$; $P < 0.05$) negative correlation exists in long-duration, and a significant ($R^2 = 0.957$; $P < 0.05$) positive correlation in medium-duration varieties (data not shown) between yield and disease. Even though the correlation was found to be significant in both cases, it would be advisable to use more varieties before firm conclusions can be drawn in this regard. The negative correlation for long-duration varieties suggests that high yield losses are associated with increasing disease severity over time.

In sprayed plots, the fungicides, flusilazole/carbendazim and azoxystrobin, effectively controlled *U. cajani* and possibly Cercospora leaf spot. Using the rating system, no signs of disease were recorded for these plots during the two assessments, despite chlorotic flecks on the leaves confirming the presence of the pathogen. Spraying the fungicides alternately, as suggested by Syngenta (2001), to prevent pathogens from developing resistance to azoxystrobin, was highly effective in controlling rust. Punch-Xtra® is registered against rust, caused by *Puccinia arachidis* Speg. on groundnuts (Thomson, 1991; Du Pont, 1994). Dunhin (2001) showed azoxystrobin to be sufficiently effective against *Puccinia sorghi* Schwein (common rust) on maize. Other fungicides of the strobilurin group have been registered against rust of legumes, for example Headline™ EC fungicide (BASF, 2002). The fungicides applied in the present study have the advantage of being systemic and being effective against a variety of leaf spots, including *Cercospora* spp. (Thomson, 1991; Du Pont, 1994; Syngenta, 2001). *Cercospora cajani* Hennings is known to cause serious losses in pigeonpea (Reddy *et al.*, 1993).

When aiming for integrated disease control, a range of control tactics are required to suppress disease below damaging economic thresholds without damaging the agroecosystem (Lewis and Papavizas, 1988). However, contrary to what was expected, ICP 6927, while having the highest disease incidence, also

had the highest 100-pod seed mass. The control of pigeonpea rust with fungicides therefore did not significantly ($P < 0.05$) affect yield, and is not an effective control measure in itself. However, when selecting fungicides for control of other pigeonpea diseases, it is advisable to choose those that can inhibit rust, as a precaution. The best control method for pigeonpea rust is therefore host resistance, despite results of the present study which suggests that *U. cajani* is of minor importance to pigeonpea cultivation in South Africa. Further studies to evaluate pigeonpea varieties for resistance to *U. cajani* are therefore required.

The rating system developed in the present study for the pigeonpea-rust pathosystem, is of significant importance when determining the influence of the pathogen on the host plant. It can also be used effectively to study environmental influences on the host-pathogen system, by comparing the disease severity of similar varieties planted in different areas.

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Figure 4.1. A: *Uredo cajani* pustules on the abaxial leaf surface of *Cajanus cajan*. **B:** Rust observed in field trials in Mpumalanga, 2001.

Figure 4.2. The modified scale for evaluation of rust on *Cajanus cajan* varieties with different growth habits.

Key to use of figure 4.2:

Inspect zone 1,

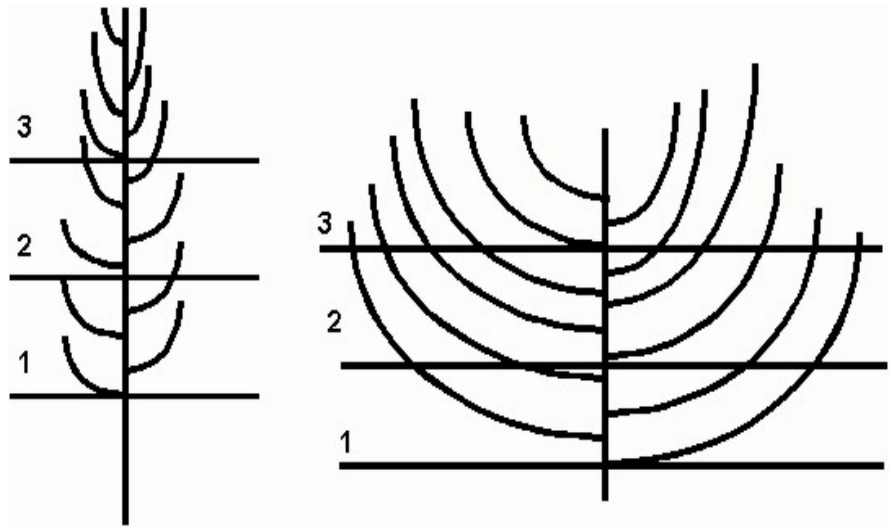
- If no disease is visible, the rating is 0.
- If zone 1 is diseased, move on to zone 2.
- If zone 2 is not diseased, rate disease in zone 1 as A, B, C or D using figure 4.2 and table 4.1.
- If zone 2 is diseased, move on to zone 3.
- If zone 3 is not diseased, rate disease in zone 2 as A, B, C or D using figure 4.2 and table 4.1.
- If zone 3 is diseased, rate disease in zone 3 as A, B, C or D using figure 4.2 and table 4.1.

Figure 4.3. Mean disease ratings for rust on *Cajanus cajan*, assessed in April and May 2002 on three medium-duration (ICP 6927, ICPL 87051 and ICPL 87119) and three long-duration varieties (ICEAP 0020, ICEAP 0040 and ICEAP 0053) in Mpumalanga. Assessments were made for treated (T) and untreated (UT) plots. Standard deviation is represented by error bars.

Figure 4.4. Performance of three medium-duration (ICP 6927, ICPL 87051 and ICPL 87119) and three long-duration pigeonpea varieties (ICEAP 0020, ICEAP 0040 and ICEAP 0053) in Mpumalanga, 2001/2002. Performance was determined for plots treated alternately with flusilazole/carbendazim and azoxystrobin, and untreated plots which received no fungicide applications. Standard deviation is represented by error bars.

A

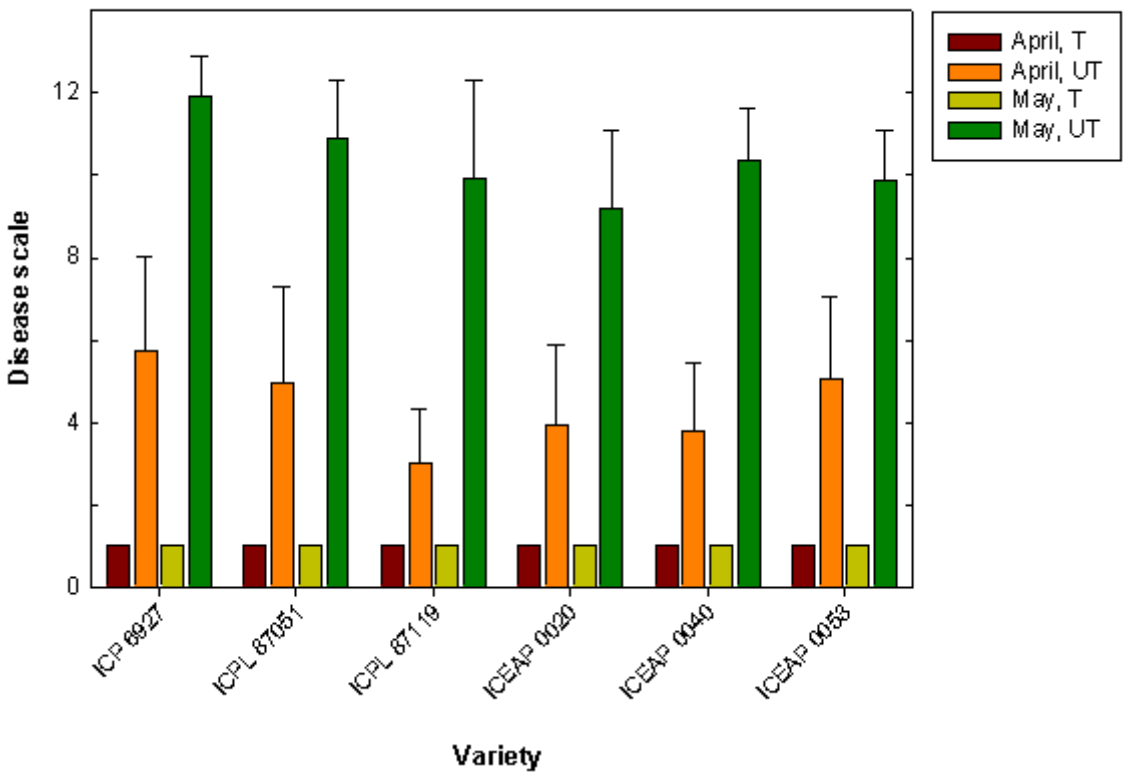
B



Erect/compact **Spreading**

A **B**

C **D**


April, T
April, UT
May, T
May, UT
Disease scale
0
4
8
12
ICP 6927
ICPL 87051
ICPL 87119
ICEAP 0020
ICEAP 0040
ICEAP 0053
Variety

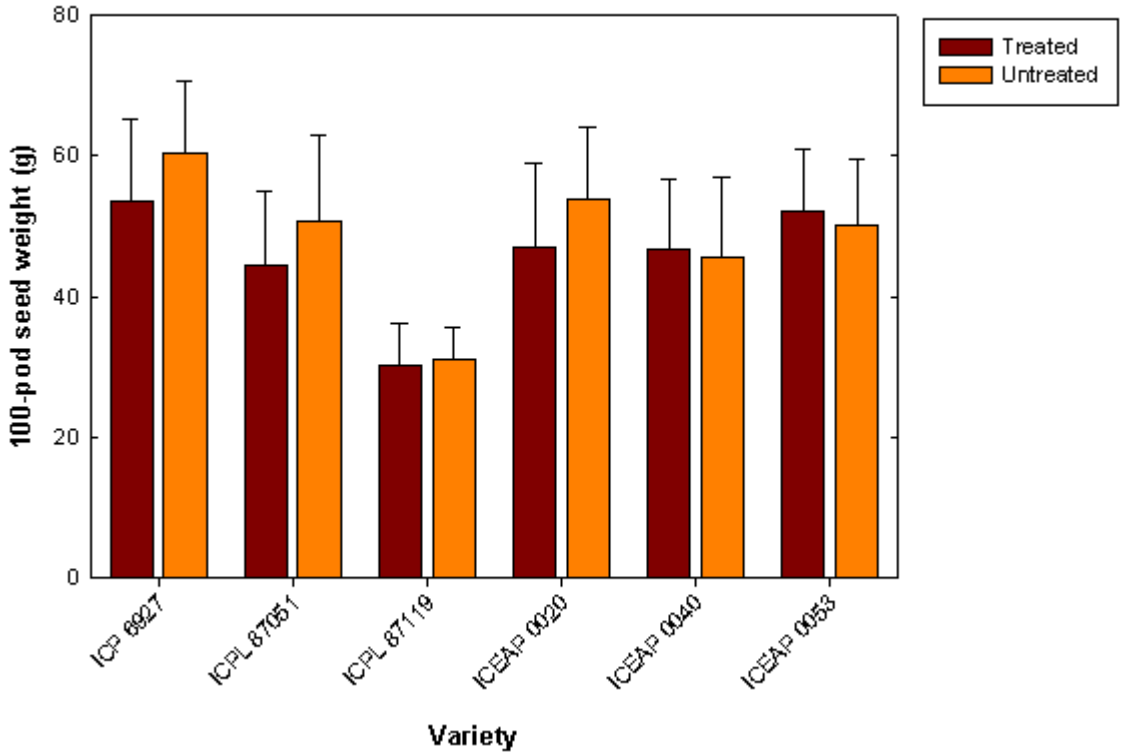

Treated
Untreated
100-pod seed weight (g)
0
20
40
60
80
ICP 6927
ICPL 87051
ICPL 87119
ICEAP 0020
ICEAP 0040
ICEAP 0053
Variety

Table 4.1 A 3-point scale used for field evaluation of rust caused by *Uredo cajani* in pigeonpea

Zone	Rating**	Disease observed
	0	No disease observed
1	A	Less than 60 rust pustules on first 2 leaves of primary branch
	B	Pustules on the first 3rd of the branch
	C	Pustules on two thirds of the branch
	D	The whole primary branch has pustules
2	A	Less than 60 rust pustules on first 2 leaves of primary branch
	B	Pustules on the first 3rd of the branch
	C	Pustules on two thirds of the branch
	D	The whole primary branch has pustules
3	A	Less than 60 rust pustules on first 2 leaves of primary branch
	B	Pustules on the first 3rd of the branch
	C	Pustules on two thirds of the branch
	D	The whole primary branch has pustules

* Table 4.1 has to be used in conjunction with figure 4.2 to assess disease in the field.
** The letter corresponds with the letter in figure 4.2, to illustrate the pustule progress and distribution on seperate branches.

SUMMARY

In the literature review, an overview of host, pathogen and environment related factors, showed that by studying sub-components of the disease triangle individually, and then integrating them, a holistic approach to control of pigeonpea (*Cajanus cajan*) diseases is possible. Sub-components that were identified as being most important in the control of legume diseases, were host resistance and cultural practices.

Two fungal species, consistently isolated from pigeonpea leaf spots, were identified as *Passalora cajani* and *Cercospora apii*. Pathogenicity could however not be proven for these species, suggesting that they are probably latent-infecting fungi. The *C. apii* and *P. cajani* isolates varied significantly regarding nutritional and temperature requirements. Inhibition by fungicides varied, with flusilazole/ carbendazim inhibiting growth of both *P. cajani* and *C. apii* most effectively, but mancozeb was found to be least effective. The general trend was that all five fungicides inhibited fungal growth effectively at concentrations higher than 5 µg a.i./ ml. Cluster analysis using physiological data revealed two distinct groups, one group containing the three *C. apii* isolates, and the other group the eight *P. cajani* isolates. Results of a molecular study were consistent with those of physiological studies.

Development of infection structures were studied for the pathogen *Uredo cajani*, cause of pigeonpea rust. *Uredo cajani* was shown to be a well adapted parasite, with leaf topography influencing the growth habit of germ tubes and infection. A comparison of infection structures, at different sampling times and on six pigeonpea varieties, showed some variation in susceptibility which was of a non-hypersensitive nature. This mechanism seems to be sufficient to restrict *U. cajani* infection, and probably explains why pigeonpea rust is considered of lesser importance. Variety ICP 6927 was least and ICPL 87119 most susceptible to rust. Susceptibility levels in medium-duration (MD) varieties were variable, but more consistent in long-duration (LD) varieties.

A rating system was developed which provided a simple and effective aid in determining varietal and treatment differences based on the severity of rust on

pigeonpea. Higher disease incidence was observed in MD varieties, due to the shorter maturation period when compared to LD varieties. All varieties used in this trial were susceptible to rust, with variety ICP 6927 most susceptible and ICEAP 0020, least susceptible. Flusilazole/carbendazim and azoxystrobin, sprayed alternately, effectively controlled *U. cajani*, but fungicide treatments did not significantly increase seed yield nor quality. The highest yielding variety was ICP 6927 and the lowest ICPL 87119. A negative correlation for LD varieties between yield and disease suggests that high yield losses are associated with increasing disease severity over time and the control of rust on pigeonpea with fungicides is an effective, but not economically viable, control measure.

OPSOMMING

In die literatuuroorsig van gasheer-, patogeen- en omgewingsverwante faktore, was dit duidelik dat ‘n studie van die individuele subkomponente van die siektedriehoek tot ‘n holistiese benadering vir die beheer van duifert-siektes kan lei. Subkomponente wat as belangrik geïdentifiseer is in die beheer van peulgewassiektes, is gasheerweerstand en verbouiingspraktyke.

Twee swamspesies, wat gereeld vanaf blaarvlek geïsoleer is, is geïdentifiseer as *Passalora cajani* en *Cercospora apii*. Patogenisiteit kon nie vir hierdie isolate bewys word nie, wat op ‘n moontlike latente infeksietipe dui. Die *C. apii* en *P. cajani* isolate het betekenisvol verskil in hulle voedings- en temperatuurvereistes. Inhibisie van swamgroei deur fungisiede het ook grootliks verskil. Die fungisied flusilasol/karbendazim het die groei van beide spesies die doeltreffendste geïnhibeer, terwyl mankoseb ondoeltreffend was. Oor die algemeen het al vyf fungisiede swamgroei doeltreffend geïnhibeer teen konsentrasies bo 5 µg a.b./ml. Groeperingsanalise, gebaseer op fisiologiese data, het twee groepe uitgewys wat onderskeidelik *C. apii* en *P. cajani* isolate bevat. Resultate van ‘n molekulêre studie het met fisiologiese data ooreengestem.

Die ontwikkeling van infeksiestrukture van die patogeen *Uredo cajani*, oorsaak van roes op duifert (*Cajanus cajan*), is beskryf. Daar is gewys dat *U. cajani* ‘n goed aangepaste patogeen is en dat blaartopografie die groeiwyse van kiembuise en infeksie kan beïnvloed. Deur infeksiestrukture op ses duifert-variëteite te vergelyk, is variasie in vatbaarheid, van ‘n nie-hipersensitiewe aard, bevestig. Hierdie meganisme mag voldoende wees om die verspreiding vanaf die infeksiepunt van *U. cajani* te beperk en is ‘n moontlike verduideliking waarom hierdie siekte nie as belangrik beskou word nie. Variëteit ICP 6927 was die vatbaarste, en ICPL 87119 die minste vatbaar. Vatbaarheidsvlakke het gevarieër in die medium-durasie (MD) variëteite, maar was meer konsekwent in die lang-durasie (LD) variëteite.

‘n Evalueringsisteem, wat ontwikkel is om verskille tussen variëteite gebaseer op siektevoorkoms aan te dui, het eenvoudige en doeltreffende lesings tot gevolg gehad. Die waargenome siektevoorkoms was hoër in die MD as in die

LD variëteite, as gevolg van ‘n korter groeiperiode. Al ses variëteite was vatbaar vir roes, met variëteit ICP 6927 die vatbaarste en ICEAP 0020 die minste vatbaar. Flusilasol/karbendazim en asoksistrobien, alternatief gespuit, het *U. cajani* effektief beheer, maar het nie die saadgehalte of -kwaliteit betekenisvol verhoog nie. ICP 6927 het die hoogste en ICPL 87119 die laagste opbrengs gehad. ‘n Negatiewe korrelasie tussen siekte en opbrengs in die LD variëteite, dui moontlik daarop dat hoë opbrengsverliese geassosieer kan word met toename in siektegraad oor tyd. Die beheer van roes op duifert met behulp van fungisiede is doeltreffend, maar nie ekonomies regverdigbaar nie.