

**DAMPING-OFF AND STEM ROT  
OF COWPEA IN BENIN CAUSED  
BY *SCLEROTIUM ROLFSII***

by

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Submitted in partial fulfilment  
of the requirements  
for the degree of

Doctor of Philosophy

In the Faculty of Natural and Agricultural Sciences  
Department of Microbiology and Plant Pathology

University of Pretoria

Pretoria

June 2004

**DECLARATION LETTER**

I, the undersigned, declare that the thesis, which I hereby submit for the degree of Doctor of Philosophy at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at this or any other tertiary institution.

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Appolinaire Adandonon

June 2004

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**Damping-off and stem rot of cowpea in Benin caused by *Sclerotium Rolfsii***

By

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**Abstract**

The damping-off and stem rot disease syndrome is harmful to many cultivated crops. Damping-off and stem rot caused by *Sclerotium rolfsii* Sacc. on cowpea results in yield losses with serious socio-economic implications. The objectives of the present study were to investigate the occurrence of the diseases in Benin, study etiology and factors influencing the diseases, and develop strategies for the control of the diseases in the field. Results showed that the diseases are distributed countrywide. *Sclerotium rolfsii* was the main causal agent but minor pathogens, namely *Pythium* sp., *Rhizoctonia solani* Kühn and *Phoma pomorum* Thüm were also recorded. In the Ouémé valley, the diseases were favoured by soil moisture and *S. rolfsii* initial inoculum that were higher closer to the river. *Sclerotium rolfsii* isolates collected in the valley showed genetic diversity in terms of pathogenicity, mycelial compatibility groups and ITS rDNA sequences. A paper-based screening method was found to be a rapid laboratory method for screening for resistance in cowpea cultivars. Furthermore, *Moringa oleifera* L. leaf extracts, *Trichoderma* Kd 63 and *Trichoderma* IITA 508 significantly reduced the disease incidence. The best disease control was recorded in the field when *M. oleifera* seed treatment was integrated with a soil sprinkle of *Trichoderma*. The present work provides information on damping-off and stem rot of cowpea in Benin and control strategies for ecologically sustainable cowpea production.

Keywords: Biocontrol, cowpea, damping-off and stem rot, genetic diversity, integrated control, ITS rDNA, *Moringa oleifera*, polyphenols, screening, *Sclerotium rolfsii*, *Trichoderma*

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ACKNOWLEDGMENTS

I would like to thank the following people who contributed to the completion of this study:

Prof T. A. S. Aveling for supervising this project, for scientific advice and solid grounding you gave me during my Master's and the current degrees. I learnt very much from you.

Dr M. Tamo for co-supervising this project, for scientific advice and encouragement.

Prof N. Labuschagne, Mr A. van der Merve, Dr T. Regnier and Dr G. Sanders for support.

The personnel and postgraduate students of the Department of Microbiology and Plant Pathology, University of Pretoria, for providing a good working atmosphere and support.

The International Institute of Tropical Agriculture (IITA) for financing the study.

Dr P. Neuenschwander, Dr O. Coulibaly, IITA-Cotonou, Benin, Dr C. Okafor, E. Oyewole IITA-Ibadan and Dr W. N. O. Hammond for their valuable support, advice and encouragement.

Dr D. Y. Arodokoun, DG-INRAB, Cotonou, and Dr J. Détongnon, MAEP, Cotonou, Benin, for their scientific and valuable advice, support and encouragement.

Dr R. S. Vodouhe, for his valuable advice, support and encouragement.

The National Institute of Agricultural Research of Benin (INRAB) for support.

All PRONAF-IITA and PRONAF-BENIN staff for their support and help.

Dr B. Ahohuendo, C. Agli, B. L. Kakpo-Zannou and A. Coffi, for advice and support.

My Father, Mr Ganvoesse S. Adandonon, peace to his soul.

My mother, Mrs Tohossi Bonongue, for her love. May this thesis be the fruits of your loyal and permanent support, and motivation.

Mrs Victoire Adandonon, my spouse, for her love, patience and courage. May this PhD thesis be the results of our joined efforts.

My children, Aston Vestris, Margareth Laurencia and Alvin Fresnel Adandonon, for their love, patience, courage and support. May this study be an example for you to do better.

My Uncles, particularly Mr Nestor Y. Holotchi and my brothers and sisters, especially Messrs Casimir, Albert and Ms Georgette Adandonon for their love, advice, and support.

May, all my parents, colleagues and friends receive here my deep gratitude.

Dr Jules Adé and his wife, Mrs Florence Adé for support and encouragement.

Quenton Kritzinger, R. Swart, Dr Yolisa P. Pakela, F. Hountondji, H. Baimey, R. Adéoti, R. Allomasso, N. Hounkpè, C. Agboton, T. Houndété, A. Massénon, A. Koudji, G., N. Adandonon, S. Téli and Mss Victorine Houndjo, and S. R. Holotchi for support.

The Embassy of Benin and particularly Mr Boniface and Mrs Béatrice G. Nouatin for their good friendship, encouragement and support.

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# **CHAPTER 1**

## **GENERAL INTRODUCTION**



## CHAPTER 1

### GENERAL INTRODUCTION

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Cowpea, (*Vigna unguiculata* (L.) Walp) has a great potential for increasing food legume production (Singh *et al.*, 1997). It is widely grown in Africa, India and Brazil (Singh and Rachie, 1985). Cowpea seeds and its tender leaves are eaten. Cowpea provides considerable protein (Singh and Rachie, 1985) and as a food legume it constitutes the natural protein supplement to staple diets, and represents the legume of choice for many populations in Africa (Elias *et al.*, 1964; Rachie and Roberts, 1974; Bliss, 1975). It is often called "meat for poor people", since this protein is the cheapest (Atachi *et al.*, 1984).

In spite of its nutritional and agronomical importance, cowpea yield is low due to pests and many diseases including damping-off and stem rot. The diseases were reported in USA, Brazil, Tanzania, Nigeria (Singh and Rachie, 1985) and South Africa (Adandonon, 2000; Aveling and Adandonon, 2000). In Nigeria, the diseases were reported to be caused by a complex of fungi including species of *Pythium*, *Phytophthora*, *Colletotrichum* and *Sclerotium* (Singh and Rachie, 1985).

In the Ouémé valley, Benin, damping-off and stem rot diseases constitute the major constraint to cowpea production (PEDUNE-BENIN, 1995). The diseases are caused by *Sclerotium rolfisii* Sacc. (Adandonon, 2000; Kossou *et al.*, 2001). There are no reports on the distribution of cowpea damping-off and stem rot and the associated causal fungi throughout the cowpea producing areas in Benin. More information on the factors influencing the diseases over seasons and on the strategies to control the diseases in Benin have yet to be reported.

The main objectives of the present study were to investigate the occurrence of damping-off and stem rot in Benin, study the factors favouring the functioning of the disease triangle, environment-*Sclerotium*-cowpea, leading to damping-off and stem rot expression over (at least) two seasons, and finally develop control strategies to reduce the incidence of the diseases in the field.

To achieve the above objectives, the work focused on the following aspects:

- . Conduct a survey throughout Benin to identify areas with the presence of damping-off and stem rot and identify the main causal agent(s)
- . Study over two seasons (years), pathogen initial inoculum, field environmental factors such as air temperature, relative humidity and soil moisture which may influence the development of damping-off and stem rot in the valley
- . Characterise *S. rolfsii* isolates collected from the valley in order to determine the genetic relatedness among them
- . Identify a method for rapid screening of cowpea cultivars for resistance to the diseases
- . Study possible phenolics involved in cultivar tolerance to the disease
- . Evaluate field efficacy of *Moringa oleifera* extract and biocontrol agents, *Bacillus subtilis* and *Trichoderma harzianum* for control of the diseases caused by *S. rolfsii* in the field

The present study consisted of different chapters each written according to the instructions of the particular journal the chapter was submitted to for publication:

**CHAPTER 2.** The chapter is a literature review that focuses on taxonomy, origin, production, constraints and importance of cowpea and some fungi causing damping-off and stem rot of cowpea. Aspects such as epidemiology, *S. rolfsii* isolate genetic relatedness and control strategies have also been included.

**CHAPTER 3.** A countrywide survey of Benin was conducted and a record of the areas affected, incidence of cowpea damping-off and stem rot and associated fungi was made.

**CHAPTER 4.** Two-year monitoring of damping-off and stem rot was conducted in the Ouémé valley, Benin and environmental factors influencing the diseases were recorded.

**CHAPTER 5.** *Sclerotium rolfsii* isolates collected from different areas in the valley were tested for pathogenicity and mycelium compatibility. Each isolate was subjected to ITS rDNA region as well as gel electrophoresis and DNA sequencing. Correlations among isolate mycelium compatibility groups (MCGs), pathogenicity and DNA sequences were made to determine the genetic diversity among isolates.

**CHAPTER 6.** Two methods were evaluated in the laboratory for screening of cultivars for resistance to *S. rolfsii*. All the cultivars were tested in the greenhouse and field

naturally infected with *S. rolf sii*. The results from laboratory (incidence and severity), greenhouse and field were correlated to determine whether the laboratory screening technique could be used as a rapid method to screen cowpea cultivars for resistance to *S. rolf sii*.

**CHAPTER 7.** Depending on the resistance level of cultivars, the concentration of phenolic compounds may differ and the antifungal activity varies accordingly. Cowpea cultivars with different levels of susceptibility/tolerance to damping-off and stem rot were used in the study. Cowpea stems from each cultivar were cut into pieces and polyphenolic compounds involved in the susceptibility/resistance to the disease were identified

**CHAPTER 8.** Different extracts of *Moringa oleifera* leaves were evaluated not only on *S. rolf sii* growth on PDA, but also on the diseases caused by the fungus in the greenhouse and field. Results were analysed for the effectiveness of the extracts in the field.

**CHAPTER 9.** Effectiveness of *Moringa oleifera* and formulations of *Trichoderma* and *Bacillus* in the integrated control of the diseases were evaluated in the greenhouse and field. Cowpea cultivars were inoculated with *S. rolf sii* in the greenhouse and treated with *M. oleifera* extracts, biocontrol agents and various combinations. All the treatments were also tested in the naturally infected field.

**CHAPTER 10.** Issues from each chapter are discussed.

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## **CHAPTER 2**

# **LITERATURE REVIEW**

## CHAPTER 2

### LITERATURE REVIEW

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#### 2.1 Cowpea: Taxonomy, origin and growth habit

Most nomenclatural problems of cultivated cowpeas and related wild species have recently been satisfactorily resolved (Verdcourt, 1970; Summerfield *et al.*, 1974; Westphal, 1974; Maréchal *et al.*, 1978). It is agreed that cowpeas belong to the botanical species *Vigna unguiculata* (L.) Walp. (Singh and Rachie, 1985). *Vigna unguiculata* is a member of the Order Leguminosales, Family Fabaceae, Tribe Phaseolae and Subtribe Phaseolinae (Kochhar, 1981; Singh and Rachie, 1985). It consists of one subspecies *V. unguiculata* subsp. *unguiculata* with three cultivated cultigroups: Unguiculata, Biflora and Sesquipedalis and two wild varieties (Maréchal *et al.*, 1978). Cultigroup Unguiculata is the most diverse of the cultivated subspecies *unguiculata* and has the widest distribution (Singh and Rachie, 1985). It is commonly called cowpea and is widely grown in Africa, India and Brazil (Singh and Rachie, 1985). It is the most cultivated legume in the Republic of Benin (Atachi *et al.*, 1984).

All evidence points to cowpea originating in Africa, although where the crop was first domesticated is uncertain (Singh and Rachie, 1985). Cowpea was thought to have originated in West Africa (Rawal, 1975) in an area encompassing the savannah region of Nigeria, southern Niger, part of Burkina Faso, northern Benin, Togo and the north-western part of Cameroon (Ng and Machéral, 1985). Padulosi *et al.* (1990) reported that southern Africa is the centre of genetic diversity because the most primitive of the wild cowpea occurs in Namibia from the west, across Botswana, Zambia, Zimbabwe and Mozambique to the east, and the Republic of South Africa and Swaziland to the south. The former Transvaal (Northern Province) in South Africa is depicted as the most probable centre of speciation of cowpea due to the presence of wild varieties such as var. *rhomboidea*, var. *protracta*, var. *tenuis* and var. *stenophylla*, all of which occur from the Northern Province to Cape Town, Swaziland, Zimbabwe and Mozambique (Singh *et al.*, 1997).

Cowpea is a single crop species, but the varietal requirements in terms of plant type, maturity, seed type, colour preference and use pattern are extremely diverse from region to

region (Singh *et al.*, 1997). The growth-habit traits of cowpea are moderately heritable (Singh *et al.*, 1997) and the life cycle depends on the cultivar (Singh and Rachie, 1985). It can be an annual or perennial crop, erect, trailing or climbing with striate smooth or slightly hairy stems (Singh and Rachie, 1985; Fox and Young, 1982). The leaves are trifoliate and the leaflets are ovate or lanceolate with entire margins or 3-lobed at the base (Fox and Young, 1982). Both flower size and style length are heritable (Emebiri, 1989) and the inflorescence is axillary with some white and mauve flowers measuring 15-22 mm long; the fruit is an erect, linear-cylindrical, smooth or slightly warty pod measuring 5-15 cm, depending on the cultivar. The seed colour ranges from white to dark red or black, often mottled, oblong or reniform (Fox and Young, 1982). The optimum temperatures for cowpea seed germination range from 20 to 30 °C (Quass, 1995). Most cowpea cultivars in the tropics and subtropical regions of Africa are grown in humid regions with an annual rainfall varying from 1500-2000 mm (Tindal, 1983). The future developmental plant vigour depends on the cultivar and Ogunbodede (1988) reported considerable genetic variability in cowpea for several seedling traits.

## 2.2 World cowpea production

In 1981, the world cowpea production area was estimated at 7.7 million hectares with an annual grain production of 2.27 million tonnes (Singh and Rachie, 1985; Table 2.1).

**Table 2.1**

Major cowpea-producing countries and estimated production (1981)  
(Singh and Rachie, 1985)

Country	Production (t)
Nigeria	850,000
Brazil	600,000
Niger	271,000
Burkina Faso	95,000
USA	60,000
Ghana	57,000
Kenya	48,000
Uganda	42,000
Malawi	42,000

The major producers included Nigeria, Niger, Brazil, Burkina Faso, Ghana, Kenya, Uganda and Malawi (Singh and Rachie, 1985; Table 2.1). Rapid progress in cowpea improvement has been made and the increase of cowpea production in traditional growing regions has resulted in cowpea expansion into new areas (Singh and Rachie, 1985). Cowpea is widely distributed throughout the tropics and recently, the world cowpea production was estimated at 3 million tonnes grown on a global production area of 12.5 million hectares, with 64 % (8 million hectares) of this total in West and Central Africa followed by about 2.4 million hectares in Central and South America, 1.3 million hectares in Asia, and about 0.8 million hectares in East and southern Africa (Singh *et al.*, 1997).

## 2.3 Cowpea in the Republic of Benin

### 2.3.1 Cultural practices

The Republic of Benin is a West African Country bordered in the north by Niger and Burkina Faso, in the south by the Atlantic Ocean, in the east by the Federal Republic of Nigeria and in the west by Togo Republic. There are six Departments in Benin: Atacora, Borgou, Zou, Mono, Atlantique and Ouémé (MDR, 1999). Cowpea production for the last ten years (Table 2.2) shows an increase in the total annual production (MDR, 1999).

**Table 2.2**

Cowpea production in the Benin Republic (1987-1996) (MDR, 1999)

Years	Production (t)
1987	42,500
1988	44,100
1989	48,800
1990	47,900
1991	55,200
1992	62,200
1993	58,200
1994	65,000
1995	62,900
1996	64,200



Cowpea is cultivated throughout the country (Table 2.3). In general, the cultural practices vary from one cowpea production area to another and farmers prepare soil differently before sowing (PEDUNE-BENIN, 1995). The cowpea planting season is variable and depends on the areas, the objectives of the farmers and the varieties to be sown (PEDUNE-BENIN, 1995). In the north, cowpea is sown in June-July. If the objectives of the farmers are to produce cowpea as a vegetable or fodder for animals, it is sown anytime during the rainy season, even between sorghum rows regardless of the age of the sorghum plant (PEDUNE-BENIN, 1995). In the central and southern part of the country the cowpea planting season is in May (long rainy season) and in August (short rainy season). The planting date for cowpea production in the Ouémé valley is in November-December (PEDUNE-BENIN, 1995).

**Table 2.3**

Cowpea production in 1996 per Department in the Republic of Benin (MDR, 1999)

Department	Production (t)
Atacora	10,700
Borgou	12,100
Zou	19,700
Mono	7,300
Atlantique	2,800
Ouémé	11,500

Cowpea is sown at 2, 3 or 4 seeds per drill. Spacing between plants varies and depends on the area and the other crops in association with cowpea (PEDUNE-BENIN, 1995). Cowpea production is more important in the central and southern parts of the country (Table 2.3) and it is generally grown as sole crop but can be mixed or in rotation with, or grown as relay crop after other crops such as maize (PEDUNE-BENIN, 1995). Sometimes, it is grown in the early development stage of oil palm fields as in the Mono Department where the soil is poor and land is a constraint with little or no fallow period (PEDUNE-BENIN, 1995).

Farmers rarely fertilise a cowpea field except for part of the Mono Department where the soil is poor in organic matter (PEDUNE-BENIN, 1995). This fertilisation allows

good growth of cowpea in this soil (Ezedinma, 1961; Agboola, 1978; Coulibaly, 1984; Singh and Rachie, 1985). In contrast, in the Ouémé valley, the soil is not fertilised but always fertile because straw of maize and weeds are often left on the farms and rot after flooding (PEDUNE-BENIN, 1995). Soil preparation only consists of cutting weeds and making holes in the soil where cowpea seeds are directly sown.

### 2.3.2 Cowpea constraints

Among the major constraints limiting cowpea production in Benin are striga, rodents, insect pests and diseases. *Striga gesnerioides* (Wild.) Vatke (called witchweed) belongs to the family Scrophulariaceae and is a parasitic plant attacking cowpea in the field (Kuijt, 1969). *Striga gesnerioides* is widely distributed (Singh *et al.*, 1997) and it is widespread in the northern and central parts of Benin (PEDUNE-BENIN, 1995) where there is no successful control.

During the life cycle of cowpea, rodents and insect pests are major problems throughout the country and yield losses are often up to 100 % if not controlled (Singh and Rachie, 1985). Rodenticides are available but economically unaffordable for small-scale farmers (Singh and Rachie, 1985). The Benin Crop Protection Service suggests farmers use cyfluthrin-malathion (“kinikini” in local language) and other chemicals to control insect pests. Chemicals to control pests are always expensive, rare and sometimes ineffective because they have expired (PEDUNE-BENIN, 1995). The yield is often very low at farmer level and is around 300-600 kg ha<sup>-1</sup> (PEDUNE-BENIN, 1995). In storage, insect pests, mainly *Callosobruchus* spp., damage cowpea seeds and often results in 100 % losses after six months. Farmers control them by mixing cowpea seeds with pepper powder, groundnut (*Arachis hypogea* L.) oil, wood ash and by putting them in a metal barrel (as is the case of Ouémé valley and part of Mono) or in other containers in the other Departments (PEDUNE-BENIN, 1995). Benin has been involved, since 1994, in a regional project initiated by the International Institute of Tropical Agriculture (IITA), namely Ecologically Sustainable Cowpea Plant Protection (French acronym: PEDUNE). The project developed, advised and/or tested the use of natural products such as leaves and seeds of neem (*Azadirachta indica* A. Juss) and papaya (*Carica papaya* L.) to control pests and diseases in the field (PEDUNE-BENIN, unpublished). In storage, solar heating with clear and black polyethylene and double bagging is the common control measure, as suggested by the project and applied by farmers to control storage pests (PEDUNE-BENIN, unpublished).

Cowpea diseases encountered in Benin are mostly caused by viruses, bacteria and fungi (PEDUNE-BENIN, 1995). Among viral diseases, mosaic viral diseases are the most often encountered in Benin. Most of the varieties grown in Benin, but not all, escape the damages caused by viral diseases and succeed in producing, to some extent, an acceptable yield. There are no specific measures to control viral diseases in Benin. Bacterial blight and pustule are the major bacterial diseases (Singh *et al.*, 1997). Bacterial blight disease caused by *Xanthomonas campestris* (Burkholder) Dye occur in Benin and especially in the northern part where some yield losses are in excess of 44 % at experimental levels (Wydra and Sikirou, 1997a). The pathogen is seed-borne. The severity of the disease can be reduced in mixed cultivation with cassava or maize (Wydra and Sikirou, 1997b). The major fungal diseases of cowpea include anthracnose, ascochyta blight, black leaf spot or leaf smut, brown blotch, brown rust, cercospora and pseudocercospora leaf spots, powdery mildew, septoria leaf spot, sphaceloma scab, web blight and damping-off and stem rot (Singh *et al.*, 1997).

The cowpea diseases that are the most destructive in the Ouémé valley, Benin, are pre- and post-emergence seedling damping-off and stem rot (PEDUNE-BENIN, 1995). These diseases are an important constraint during cowpea production after natural flooding in the valley. The diseases are so severe as the soil is still very wet during cultivation (PEDUNE-BENIN, 1995). Poor stands result from either lack of germination owing to seed rot or pre- and post-emergence seedling damping-off. The causal fungus infects the base of the seedling and the lesion girdles the hypocotyl. The fungus produces a fan of silky white mycelium and round sclerotia which are initially white and gradually darken (Singh and Allen, 1979). Cowpea yield losses range from 19 to 40 % (PEDUNE-BENIN, 1995). In 2000, diseased samples taken from some villages in the valley revealed *Sclerotium rolfsii* Sacc. as causal agent of the diseases (Adandonon, 2000). Additional research over seasons is needed to get more complete information on the diseases and effects of environmental factors on the development of the diseases in the valley. To date, there are no effective measures to control the diseases.

#### **2.4 Importance of cowpea**

Cowpea is of major importance to the livelihoods of millions of relatively poor people in less developed countries of the tropics (Singh *et al.*, 1997). From production of this crop, rural families variously derive food, animal feed, and cash together with spillover benefits to their farmlands. Cowpea grain is widely traded out of the major production areas and

provides a cheap and nutritious food for relatively poor urban communities (Singh *et al.*, 1997).

As with other food legumes, cowpea has a high protein content (Table 2.4) (Elias *et al.*, 1964; Rachie and Roberts, 1974) and constitutes the natural protein supplement to staple diets. It is often called "meat for poor people", since this protein is the cheapest (Atachi *et al.*, 1984). In fresh form, cowpea young leaves, immature pods and peas are used as vegetables, as is the case in Benin, Nigeria, east and southern Africa, while several snacks and main meal dishes are prepared from the grain. In Asia green pods are eaten whereas in East and southern Africa, the tender leaves are regularly picked and eaten as spinach (Singh *et al.*, 1997).

**Table 2.4**

Protein content of some edible legumes compared to protein content of cereals  
(Aykroyd *et al.*, 1982)

Legume/cereal	Protein (g 100 g <sup>-1</sup> of whole dry ripe grains)	Lysine (g 16 g <sup>-1</sup> nitrogen)	Méthionine (g 16 g <sup>-1</sup> nitrogen)
<i>Arachis hypogea</i>	23.4	-	-
<i>Phaseolus vulgaris</i>	20.3	-	-
<i>Macrotyloma geocarpum</i>	20.2	-	-
<i>Cajanus cajan</i>	20.4	-	-
<i>Glycine max</i>	35.1	-	-
<i>Vigna unguiculata</i>	28.9	6.1	1.3
<i>Cicer arietinum</i>	19.4	-	-
Cereals (e.g. <i>Oryza sativa</i> )	6.4	3.6	2.1

One of the most important nutritional characteristics of food legumes, including cowpea, is that they complement cereal grains (Bressani and Scrimshaw, 1961). Starchy roots, tubers, cereal grains and fruits, because they are eaten in large quantities, provide considerable protein but the nutritive value of the latter is not sufficient, particularly for children and pregnant and lactating women (Singh and Rachie, 1985). The protein quality is synergistically improved in cereal-legume mixes because of the lysine contributed by the cowpea and the methionine contributed by the cereal (Table 2.4)

(Sherwood *et al.*, 1954; Bressani and Scrimshaw, 1961; Bressani, 1975; Rachie and Silvestre, 1977; Aykroyd *et al.*, 1982; Singh and Rachie, 1985).

Concerning the agronomical importance, cowpea cultivars with spreading indeterminate or semi-determinate bushy growth provide ground cover, thus suppressing weeds and providing some protection against soil erosion (Singh *et al.*, 1997). Furthermore, like many other legumes, cowpea can symbiose with nodule bacteria (rhizobia) present in most, if not all, tropical soils (Singh and Rachie, 1985). The rhizobia possess the nitrogenase complex, an enzyme capable of reducing atmospheric nitrogen (N<sub>2</sub>) into compounds assimilable by the host plant (Singh and Rachie, 1985). Effective cowpea-*Rhizobium* symbiosis fix more than 150 kg N/ha and supply 80-90 % of the host plant N requirement (Eaglesham *et al.*, 1977; Summerfield *et al.*, 1977). This attribute allows adequate yield in N-deficient soils where non-nodulated crops such as cereals fail. Maximising Nitrogen (N<sub>2</sub>) fixation is an economical way to cope with the shortage of expensive nitrogenous fertilisers in tropical countries (FAO, 1976). This biological nitrogen fixation is beneficial to subsequent cereal crops in rotation or association with cowpea (Singh and Rachie, 1985).

## **2.5 Cowpea damping-off and stem rot-causing fungi**

### **2.5.1 *Phytophthora* damping-off and stem rot**

*Phytophthora* cowpea damping-off and stem rot is of local importance in North America, Australia and India (Singh and Allen, 1979). These diseases are relatively new in Africa and it is hoped that it is still confined to Ilonga (Tanzania) where it was first observed in 1970 (Singh *et al.*, 1982). In Ilonga, out of 1781 lines screened for resistance to the diseases, only two were found to be resistant; the rest were destroyed by the diseases within 20-25 d of inoculation (Singh *et al.*, 1982). Most species of *Phytophthora* cause diseases primarily on the lower stem, and cowpea plants are susceptible to infection by *Phytophthora* spp. at all stages of growth (Purss, 1957; Singh *et al.*, 1982). Wilting of affected seedlings is rapid (Purss, 1957). According to Purss (1957) and Fernando and Linderman (1993), individual infected plants show wilting when leaves are still green and a brownish lesion starting at or near the soil surface, extending upwards. The lesion girdles the stem and results in permanent wilting and death of the plant. Internally, brown discolouration extends a short distance into healthy tissue.

### 2.5.2 *Pythium* damping-off and stem rot

*Pythium* species causing pre- and post-emergence seedling damping-off diseases on cowpea include *Pythium aphanidermatum* (Edson) Fitzp. (Singh and Allen, 1979; Koleosho *et al.*, 1987) and *Pythium ultimum* Trow (Aveling and Adandonon, 2000). The damping-off diseases result in poor stands due to seedling infections by the fungus after the seed has germinated but before the seedling has emerged above the soil line (Singh and Allen, 1979). The infection is characterised by a grey-green, water-soaked girdle of the seedling stem. The invaded cells collapse, and the seedling dies shortly after infection (Singh and Allen, 1979). The diseases at this stage is called pre-emergence damping-off (Singh and Allen, 1979). Seedlings that emerge above the soil are usually attacked at the roots and sometimes at or below the soil line. The invaded areas become water-soaked and extend from soil level upward (Onuorah, 1973; Williams, 1973). The invaded cells collapse and the plants quickly wilt, the seedling falls over on the soil and later dies. This phase of the diseases is called post-emergence damping-off (Singh and Allen, 1979). Williams (1973) reported that seedling mortality induced by the fungus resulted in as low as 25 % seedling stand in cowpea sown during cool, wet weather in Ibadan, Nigeria.

### 2.5.3 *Rhizoctonia* damping-off and stem rot

The seedling mortality caused by *Rhizoctonia solani* Kühn occurs during cool, wet weather (Singh and Rachie, 1985). The fungus attacks and kills the growing tip of the seedling, which then dies. Older seedlings may also be attacked. The lesions then girdle the stem and the plant may die. In the damping-off diseases, root lesions also form, since *Rhizoctonia* frequently attacks the roots at the same time it attacks the stem (Singh and Rachie, 1985). Infected roots have small, sunken lesions that are light to dark-brown (Singh and Rachie, 1985).

### 2.5.4 *Sclerotium* damping-off and stem rot

Damping-off symptoms are brown lesions on the hypocotyl base, early dying and falling-over on the ground of seedlings with wilting whereas those of stem rot are brown lesions on the stem base, yellowing, wilting, dying of stems remaining upright, with white mycelium in the lesions. The causal fungus infects the bases of stems producing a fan of silky white mycelium and round sclerotia which are initially white and gradually darken (Singh and Allen, 1979). When seedlings are attacked by *Sclerotium*

*rolfsii* Sacc., the fungus invades all parts of the seedlings and they die rapidly. When the fungus attacks plants that have already developed some woody tissue, it does not invade throughout, but grows into the cortex and slowly or quickly girdles the plants, which eventually die (Singh and Allen, 1979). Twenty percent mortality has been reported in India (Ramaiah *et al.*, 1976).

## **2.6 *Sclerotium rolfsii*: Nomenclature, host range and distribution**

The genus *Sclerotium* was described by Saccardo (1911) to include fungi which formed differential sclerotia and that had sterile mycelia. Many diverse fungi are included in this genus, and they generally form small, tan to dark-brown to black, spherical sclerotia, which are internally differentiated into a rind, cortex, and medulla (Singleton *et al.*, 1992). A large number of plant pathogenic fungi that formed sclerotia of this type and having no known sexual or conidial state were placed in the genus *Sclerotium*. As more became known about the fungi and their sexual states were discovered, some were placed into more appropriate genera and species (Singleton *et al.*, 1992). For example, *Macrophomina phaseolina* (Tassi) Goid. (Holliday and Punithalingam, 1970) *Rhizoctonia tuliparum* Whetzel and Arthur (Mordue, 1974a), *Rhizoctonia oryzae-sativa* (Saw.) Mordue, and *Rhizoctonia fumigata* (Nakata ex Hara) Gunnell and Webster (Gunnell and Webster, 1987) all formerly belonged to the genus *Sclerotium*. Current members of the genus *Sclerotium* may have teleomorphs that belong to the Basidiomycotina (*S. rolfsii*, teleomorph *Athelia rolfsii* (Curzi) Tu and Kimbrough) (Punja, 1985) or Ascomycotina (*Sclerotium oryzae* Cattaneo, teleomorph *Magnaporthe salvinii* (Catt.) Krause and Wester) or may have no known sexual state (*Sclerotium cepivorum* Berk.) (Coley-Smith, 1960). Other species within the genus *Sclerotium* include *Sclerotium delphinii* Welch and *Sclerotium coffeicola* Stahel which produce larger sclerotia than those of other *Sclerotium* species (Singleton *et al.*, 1992).

*S. rolfsii* is a soil-borne plant pathogen of worldwide importance with a very extensive host range including more than 500 plant species (Aycock, 1966; Punja, 1985; 1988; Singleton *et al.*, 1992; Harlton *et al.*, 1995; Cilliers *et al.*, 2000). Most *S. rolfsii* diseases have been reported on dicotyledonous hosts, but with several monocotyledonous species also being infected (Aycock, 1966; Mordue, 1974b). Hot and humid weather is conducive to sclerotial germination and mycelial growth. Consequently the diseases caused by the fungus are more serious in subtropical and tropical regions than in temperate regions (Yorinori, 1994). In low-altitude, guinea savana zones of Africa,



damping-off diseases caused by *S. rolfsii* were reported to be of minor importance (Singh and Rachie, 1985). However, *S. rolfsii* was reported to be a destructive soil-borne pathogen causing diseases on many crop plants especially in the tropics and subtropics (Mukherjee and Raghu, 1997). Growth of *S. rolfsii* is optimal at 27-30°C and sclerotia do not survive at temperatures below 0°C. This requirement limits the distribution of the pathogen to regions which have mild winters (Singleton *et al.*, 1992). The large number of sclerotia produced by *S. rolfsii* and their ability to persist in the soil for several years, as well as the prolific growth rate of the fungus (2-3 cm per d in culture) make it a well-suited facultative parasite and a pathogen of major importance throughout the world (Punja, 1988). The first confirmed report of losses due to this pathogen in the USA was made by Rolfs in 1892 on tomato (*Lycopersicon esculentum* Miller) in Florida (Aycock, 1966). Later, *S. rolfsii* diseases have been reported on cowpea in Nigeria, Brazil, USA and Asia (Singh and Rachie, 1985; Singh *et al.*, 1997). Recently, *S. rolfsii* was recorded in South Africa as causal agent of stem rot or southern blight of groundnut (Cilliers *et al.*, 2000), of bambara (*Vigna subterranea* L. Verdc.) in 2002 (Aveling, personal communication) and in Benin on cowpea (Adandonon, 2000; Kossou *et al.*, 2001).

## **2.7 *Sclerotium rolfsii*: Host tissue infection and pathogenicity**

### **2.7.1 Infection by mycelium from germinating sclerotia**

The extent of mycelial growth from sclerotia in soil is influenced by the form of germination (eruptive or hyphal) and by the presence of volatile compounds, decaying plant tissues, and soluble nutrients (Punja and Grogan, 1981; Punja *et al.*, 1984). The maximum distance from the host surface over which mycelium from an eruptively germinating sclerotium can grow and infect without an exogenous nutrient source (competence distance) is a function of many factors such as the fungal isolate, soil moisture, texture and depth (Punja, 1985). Mycelium from eruptively germinating sclerotia can infect host tissue without requiring an exogenous food base of nonliving organic matter provided they are located within the competence volume (Punja, 1985; Punja and Grogan, 1981). For hyphally germinating sclerotia to infect, a food base must indeed be provided because mycelial growth is sparse (Punja and Grogan, 1981).



### 2.7.2 Infection process and pathogenicity

A sequence of events takes place between the initial mycelial contact of the host and the maceration and death of the host tissue (Bateman and Beer, 1965; Smith *et al.*, 1984; Punja *et al.*, 1985). Although the evidence is not unequivocal, it appears that death of the host tissue occurs prior to hyphal penetration due to the production of large quantities of oxalic acid and polygalacturonases (Bateman, 1972), which act in concert (Bateman and Beer, 1965, Punja *et al.*, 1985). Mycelial aggregates that form on the host surface (Punja and Jenkins, 1984; Smith *et al.*, 1984) possibly are involved in producing these products and also may aid in penetration by exerting physical pressure. Oxalic acid sequesters calcium from the cell walls to form calcium oxalate (Punja and Jenkins, 1984) and lowers the tissue pH to the optimum for endopolygalacturonase and cellulase activity (Bateman and Beer, 1965; Bateman, 1969; 1970; 1972; Punja *et al.*, 1985). Oxalic acid is also directly toxic to plant tissue (Bateman and Beer, 1965). The pathogen also produces cellulases (Bateman, 1969; Sadana *et al.*, 1979) that may play a secondary role in tissue destruction and disease development since they appear later in the temporal sequence of enzyme production (Punja *et al.*, 1985).

Fungal hyphae grow through tissue both inter- and intracellularly. They contain numerous microbodies (Hänssler *et al.*, 1978), the presumed site of oxalate synthesis (Armentrout *et al.*, 1978) in addition to vacuoles that contain phosphatase (Hänssler *et al.*, 1975; Hänssler *et al.*, 1978). Pectic substances in host cell walls are depleted (Bateman, 1970) in advance of growing hyphae (Smith *et al.*, 1984) and peroxidase is released (Barnett, 1974) through enzymatic action. The end result is the typical water-soaked, necrotic, and macerated appearance of tissues infected by *S. rolfsii*. The production of cell-wall degradating enzymes in conjunction with oxalic acid would account, at least in part, for the extensive range of plants that succumb to infection by this pathogen (Punja, 1985). Secretion of oxalic acid and endopolygalacturonase concomitantly with rapid mycelial growth appears to be the key requirement for establishing infection (Punja *et al.*, 1985). Differences in endopolygalacturonase levels and mycelial growth rates among isolates are highly correlated with differences in virulence (Punja *et al.*, 1985).

### 2.8 *Sclerotium rolfsii*: Epidemiology

The spatial pattern of inoculum of *S. rolfsii* in naturally infested fields is clustered or clumped (Punja *et al.*, 1985; Rodriguez-Kabana *et al.*, 1974). A similar pattern has been

observed for diseased plants (Punja, 1985; Shew *et al.*, 1984). The population mean and frequency distribution of numbers of sclerotia among samples from a field are influenced by the sample probe size and field sampling pattern (Punja *et al.*, 1985). Inoculum density in fields is assessed by different approaches such as a baiting method that uses tissue segments to detect saprophytic growth (Avizohar-Hershenzon and Shacked, 1968), treating soil with methanol to stimulate sclerotial germination (Backman *et al.*, 1981; Rodriguez-Kabana *et al.*, 1980; Shew and Beute, 1984; Shew *et al.*, 1984), and direct observation and enumeration of sclerotia (Punja *et al.*, 1985; Rodriguez-Kabana *et al.*, 1974). Sclerotia may be directly recovered from soil by wet-sieving (Punja *et al.*, 1985), flotation-sieving (Rodriguez-Kabana *et al.*, 1974), or elutriation (Shew and Beute, 1984). To establish successful correlations between inoculum density and disease incidence in the field, sampling time and the location of the samples relative to the host must be considered (Punja, 1985).

Disease incidence due to *S. rolfsii* may increase following periods of temperature and moisture fluctuations; cycles of drying and wetting have been reported to stimulate germination of sclerotia (Smith, 1972; Punja and Grogan, 1981). The presence of an organic substrate for mycelial growth (senescing leaves) may enhance disease severity (Beute and Rodriguez-Kabana, 1979; Beckman and Finch, 1980; MacCarter and Kays, 1984). On carrot (*Daucus carota* L.), diseases seldom are apparent until the canopy is fully developed, which also coincides with the time at which temperatures are optimum for growth of the pathogen (Punja, 1985). Any practice bringing the inoculum into closer contact with the host can enhance disease incidence (Aycock, 1966; Gurkin and Jenkins, 1985). Punja (1985) reported that temperatures and moist conditions appear to enhance disease development regardless of whether organic matter is present.

Initial infections occur at soil level, where sclerotia are most likely to be stimulated to germinate by drying and remoistening (Punja and Grogan, 1981). Free moisture is apparently not required for infection (Punja and Jenkins, 1984). Extensive plant to plant (secondary inoculum) spread occurs in closely spaced crops such as sugar beet (*Beta vulgaris* L.) and carrot and results in the formation of disease foci (Punja, 1985). In these crops, relatively few initial foci can result in extensive disease and failure to yield. In peanut (*Arachis hypogea* L.), in which plant to plant spread is not extensive, yield is indirectly correlated with an increase in the numbers of disease foci (Rodriguez-Kabana *et al.*, 1975). Host density and the proximity of roots may thus be major factors influencing the rate of *Sclerotium* disease progression (Punja, 1985). Continuous

rotation with crops highly susceptible to the pathogen may increase disease incidence in subsequent years (Rodriguez-Kabana *et al.*, 1974).

Among factors influencing the survival of sclerotia include temperature, moisture, and biotic factors such as soil microorganisms near sclerotia (Punja, 1985). Temperatures above 50°C for extended periods are lethal to sclerotia (Porter and Merriman, 1983; Mihail and Alcorn, 1984; Yuen and Raabe, 1984). There are generally fewer sclerotia in moist than in dry soil (Bandara, 1980; Beute and Rodriguez-Kabana, 1981). However, high temperatures coupled with high soil moisture are more detrimental to survival than are high temperatures alone (Beute and Rodriguez-Kabana, 1981). Sclerotia survive better in moist soil near the surface rather than buried (Beute and Rodriguez-Kabana, 1981; Javed and Coley-Smith, 1973). Punja and Jenkins (1984) reported that death of sclerotia deeper within soil was due to increased pressure on buried sclerotia by overlying soil, which enhanced leakage and microbial antagonism, and not to lack of oxygen. Mycelium survives better in sandy soils than in fine-textured soils (Chattopadhyay and Mustafee, 1977). Factors that increase nutrient leakage and the activities of soil microorganisms or antagonism near sclerotia may accelerate the death of sclerotia (Punja, 1985). Antagonistic microorganisms such as *Trichoderma* spp. (Henis *et al.*, 1983) and *Aspergillus* spp. (Shigemitsu *et al.*, 1978) can penetrate the rind and destroy the inner sclerotial tissues. For *Trichoderma*, this is facilitated by the production of the enzymes  $\beta$ -1,3 glucanase and chitinase (Elad *et al.*, 1982; Elad *et al.*, 1983a; Elad *et al.*, 1984) and hyphae of *S. rolfsii* may be parasitised directly by *Trichoderma* spp. (Elad *et al.*, 1983b).

## **2.9 *Sclerotium rolfsii*: Sexuality and genetics**

### **2.9.1 Sexuality**

The teleomorph of the sexual state is described as *Athelia rolfsii* (Curzi) Tu and Kimbrough (Punja *et al.*, 1982). The basidial state forms on media that are poor in nutrients, in older cultures grown at low-light intensities, and following the growth of mycelium from a rich to a poor medium (Punja and Grogan, 1983a), but it is not thought to occur commonly in nature (Cilliers *et al.*, 2000). Asexual spores are not produced and the sexual stage is seldom observed (Punja *et al.*, 1982; Punja and Grogan, 1983a). Although reports of the occurrence of the basidial state are infrequent (Aycock, 1966), basidiospores can infect host tissue under greenhouse conditions and appressoria are produced from the germinating spores (Punja and Grogan, 1983a).

Reports from India indicated that aerial leaf spots caused by *S. rolfsii* (Aycock, 1966) may implicate basidiospores as a source of inoculum. Single-basidiospore strains from parental field isolates display pronounced variability in numerous characteristics, such as rate of growth, numbers of sclerotia produced, levels of cell wall degrading enzymes, and virulence (Punja and Grogan, 1983b; Punja *et al.*, 1985). Such variability following karyogamy and meiosis suggested that cells of the parental strains must have contained two or more distinct nuclear types (heterokaryotic) (Punja *et al.*, 1985). The hyphal tip cells of the single-basidiospore strains are multinucleate and clamp connections are formed at some septa (Punja, 1988). The presence of clamp connections on the hyphae makes the task of distinguishing homokaryons from heterokaryons extremely difficult without fruiting and further progeny analysis (Punja, 1988).

### 2.9.2 Genetics

#### *Variability and compatibility groups among field isolates*

Field isolates of *S. rolfsii* from various hosts and geographical areas differ in growth rate, numbers and size of sclerotia produced (Punja and Grogan, 1983b). Most isolates tend to be highly virulent (Punja *et al.*, 1985) and no asclerotial types are known from field collections. Collections made within a small geographical area, for instance a grower's field, tend to be similar morphologically, which suggests that spread most likely occurred vegetatively and not from dissemination of basidiospores (Punja, 1988). Extensive variation among isolates from a small geographical area could be used as indirect evidence of the involvement of basidiospores in spread since colonies arising from basidiospores would be highly variable (Punja, 1988).

Interest has been rekindled among plant pathologists in recent years in the use of vegetative compatibility as a technique for the identification of genetic diversity within fungal species (Singleton *et al.*, 1992). Vegetative, or heterokaryon, compatibility relies on inherent genetic characteristics of the fungus and is a very important tool for taxonomic, identification and population studies and to understand genetic relatedness of strains of fungi within a species (Singleton *et al.*, 1992). The term vegetative compatibility refers to the ability of individual strains of the same fungal species to undergo mutual asexual hyphal anastomosis, which results in viable fused cells containing nuclei of both parental strains in a common cytoplasm (Singleton *et al.*, 1992; Katan, 1998). If pairings are compatible, the hyphae intermingle, anastomosis bridges are formed from growth of lateral branches followed by fusions, the individual

mycelia cannot be discerned, and therefore, belong to the same vegetative compatibility group (VCG) (Punja, 1988). The occurrence of the same VCG on different host species or from widely different geographic areas reflects the wide host range and/or spread of the pathogen (Cilliers *et al.*, 2000). Also, the fact that isolates from within a given geographical area are diverse suggests that genetic changes have occurred within subpopulations (Cilliers *et al.*, 2000), or basidiospores may be involved in spread since colonies arising from basidiospores would be highly variable (Punja, 1988). In certain cases, isolates from the same geographic area and host plant group together (Cilliers *et al.*, 2000). Vegetative compatibility groups have been used to estimate genetic variability or relatedness within many fungal species such as *Fusarium* field populations (Puhalla, 1985; Elias *et al.*, 1993; Chulze *et al.*, 2000), *Cryphonectria parasitica* (Murrill) Barr (Powell, 1995), *Verticillium dahliae* Kleb. (Dobinson *et al.*, 1998), *Cryphonectria cubensis* (van Heerden and Wingfield, 2001), *Rhizoctonia* (Adams, 1988) and *S. rolfsii* (Punja, 1985; 1988; Singleton *et al.*, 1992; Harlton *et al.*, 1995; Cilliers *et al.*, 2000).

Early work on vegetative compatibility was done on wood rotting fungal pathogens (Punja, 1985) and hyphal interaction observation was more typical of what occurred in nature than when mutants, such as auxotrophic mutants, were employed to indirectly assess hyphal anastomosis between strains (Singleton *et al.*, 1992). The auxotrophic mutants technique for vegetative compatibility (Clarkson and Heale, 1985) requires mutagenesis followed by a selection process, and is therefore time consuming (Singleton *et al.*, 1992). Using a modification of a technique initially described for *Aspergillus nidulans* (Eidam) G. Wint. (Cove, 1976), Puhalla (1985) used nitrate-non-utilizing (*nit*) mutants, to examine vegetative compatibility relationships among several formae speciales and strains of *Fusarium oxysporum* Schlecht.: Fr.. This technique involves generating *nit* mutants on media amended with potassium chlorate (Puhalla, 1985; Correll *et al.*, 1987). Chlorate, as a nitrate analog, is reduced by enzymes in the nitrate reduction pathway, presumably forming chlorite, which is toxic (Singleton *et al.*, 1992). *Nit* mutants can usually be recovered as fast-growing chlorate-resistant sectors (Singleton *et al.*, 1992). Correll *et al.*, (1987) indicated that when two phenotypically distinct *nit* mutants from a given isolate were allowed to grow together on minimal medium containing nitrate as the nitrogen source, a heterokaryon was formed where the mycelium came in contact. The heterokaryon portion of the colony produced aerial (wild-type) mycelium due to its ability to reduce the nitrate nitrogen in

the medium. Two fungal strains capable of hyphal fusion are vegetatively compatible, and therefore, belong to the same vegetative compatibility group (Correll *et al.*, 1987; Singleton *et al.*, 1992). Similarly, Dobinson *et al.* (1998) assessed vegetative compatibility between Ontario *V. dahliae* isolates using the *nit* mutants technique.

To determine whether *S. rolfsii* isolates are compatible or incompatible, Punja (1988) indicated that isolate pairings could be made on PDA, by simultaneously placing mycelial plugs of two, three or four isolates to be tested 40 mm apart in a Petri dish and incubating for 4 to 7 d at 27-30°C. Microscopically, the formation of a zone of antagonism (or barrage zone) is the result of disruption and plasmolysis of the hyphae of both isolates, which results in thinning out of the mycelium in the zone of interaction (Punja, 1988). Harlton *et al.* (1995) and Cilliers *et al.* (2000) used this pairing method on PDA and placed *S. rolfsii* isolates in different compatibility groups. To easily detect the barrage zone between two vegetatively incompatible isolates, another method, utilising red food colouring, was first described for *Sclerotinia sclerotiotum* (Libert) de Bary (Kohn *et al.*, 1991) and was employed with *C. parasitica* (Rizwana and Powell, 1992). But the method has the disadvantage that the red food colour fades in advance of the growing mycelium of *C. parasitica* (Powell, 1995). Furthermore, the fungus was shown, in previous experiments, to rapidly lower the pH of unbuffered medium (Puhalla and Anagnostakis, 1971). Powell (1995) described experiments using other characterised pH indicators and reported that these indicators improve detection of the pH changes in mycelium of *C. parasitica*. The author tested four different pH indicators namely bromocresol green (pH 3.8, yellow; pH 5.4, blue-green), bromocresol purple (pH 5.2, yellow; pH 6.8, purple), bromophenol blue (pH 3.0, yellow; pH 4.6, blue), litmus (pH 4.5, red; pH 8.3, blue) and used red food colour as control. Powell (1995) indicated that the incorporation of the pH indicator bromocresol green (50 mg/L) in medium made mycelial cell death easier to detect and made it easier to recognise weakly barraging vegetatively incompatible strains of *C. parasitica*. The method should be applicable to other fungi, that have the capacity to lower the pH of their medium (Powell, 1995), such as *S. rolfsii* (Bateman, 1972; Punja *et al.*, 1985). Van Heerden and Wingfield (2001) utilised the bromocresol green method and detected 23 vegetative compatibility groups amongst 100 isolates of *C. cubensis*. Incompatible reactions were characterised by a dark coloured reaction when viewed from the bottom of the Petri dish, at a slight angle (van Heerden and Wingfield, 2001).



*Molecular characterisation*

There has been a rapid increase in using molecular techniques for the identification of fungi at the genus, species and subspecies level (Singleton *et al.*, 1992). Recent advances in molecular techniques have enabled inter- and intraspecific grouping of fungi (Bruns *et al.*, 1992) with few morphological differences. Molecular methods offer an entirely new approach to the diagnosis of diseases in crop plants (Lyon and Becerra-Lopezlavalle, 2000) and molecular markers are increasingly being used to characterise populations of plant pathogens (Michelmore and Hubert, 1987).

Multilocus molecular markers, including restriction fragment length polymorphisms (RFLPs) (Elias *et al.*, 1993) and random amplified polymorphic DNAs (RAPDs) based on the polymerase chain reaction (PCR) (Dobinson *et al.*, 1998) also have proven to be useful for characterising some populations of fungal pathogens. Recently, another PCR-based method, amplified fragment length polymorphism (AFLP) has been used to characterise fungal populations (Gonzalez *et al.*, 1998). A DNA amplification technique through PCR can be used not only to multiply specific regions of the DNA of any microorganism living in or around a plant (Lyon and Becerra-Lopezlavalle, 2000), but also to study fungal population biology (Arnheim *et al.*, 1990; White *et al.*, 1990). The internal transcribed spacer (ITS) region of the ribosomal DNA of fungi is one region that can be targeted in the PCR procedure (Lyon and Becerra-Lopezlavalle, 2000). Harlton *et al.* (1995) used the RFLPs-ITS region of ribosomal RNA genes (rDNA) and reported the presence of genetic variation in *S. rolfsii*.

Once a region of DNA is multiplied using PCR, the sequential order of the four constituent molecules or bases (designated by the letters A, C, G or T) in the DNA can be determined (Lyon and Becerra-Lopezlavalle, 2000). Known as DNA sequence, the order of bases in a given piece of DNA is usually unique to an organism, and can therefore act like a signature to confirm its identity. An unknown organism can thus be identified by the size or sequence of a portion of its DNA (Lyon and Becerra-Lopezlavalle, 2000). Stewart *et al.* (1999) utilised sequence data from ITS1 and ITS2 regions of the rRNA operon to separate species occurring on *Musa* and several other diverse hosts. Using sequence data, Crous *et al.* (1999) were able to separate several species occurring on *Eucalyptus*. *Rhizoctonia solani* isolates were separated based on DNA sequence and high DNA-sequence similarity was found among isolates belonging within each vegetative compatibility group, but only little similarity when different AGs were compared to each other (Adams, 1988). ITS DNA sequences have been employed

to help identify some 60 fungal isolates including *S. rolfsii* (stem and root rot) associated with cotton diseases in the field (Lyon and Becerra-Lopezlavalle, 2000). Nalim *et al.* (1995) used molecular markers and detected that all *S. rolfsii* isolates within an MCG produced identical patterns and that some MCGs shared the same patterns for the ITS region.

## **2.10 *Sclerotium rolfsii* control measures to protect cowpea from damping-off and stem rot**

Control measures include agronomic practices, use of resistant cultivars, chemical control, biological control, use of plant-derived fungicides and integrated pest-disease management (IPM).

### **2.10.1 Agronomic practices**

Control of *Sclerotium* diseases may be achieved by cultural means (Singh and Allen, 1979). The primary sources of inoculum, sclerotia in surface debris, could be reduced if plant residues are buried by deep ploughing or collected and burnt at the end of the crop season (Singh and Allen, 1979) and by soil solarisation (Punja, 1985).

### **2.10.2 Use of resistant cultivars and screening for resistance**

Fery and Dukes (1986) reported, from field experiments, that there was genetic variability in cowpea and some cultivars were more resistant to *Sclerotium* diseases. In cultivar screening trials in the Ouémé valley, Benin, variation in resistance among cowpea cultivars was recorded and K VX-396-18 followed by IT83D-326-2 were more tolerant than the others tested (PEDUNE-BENIN, unpublished).

Several compounds have been reported to be involved in plant resistance to pests and diseases (Nicholson and Hammerschmidt, 1992). Some plants are resistant to diseases caused by certain pathogens because of an inhibitory compound present in the cell before infection (Nicholson and Hammerschmidt, 1992). For example, several phenolic compounds, tannins, and some fatty acid-like compounds such as dienes, which are present in high concentrations in cells of young fruits, leaves or seeds have been proposed to be responsible for the resistance of young tissues to pathogenic microorganisms such as *Botrytis* spp. (Nicholson and Hammerschmidt, 1992).

Some phenols are inhibitors associated with nonhost resistance whereas others are formed in response to the ingress of pathogens, and their appearance is considered as



part of an active defence response (Nicholson and Hammerschmidt, 1992). Cowpeas contain polyphenols such as tannins, which are well known for reducing protein digestibility and quality (Bressani, 1985). Polyphenols are secondary metabolites distinct in their occurrence in vascular plants (Harborne, 1969). Although mainly found in the vacuoles in combination with sugars or glycosides and esters, phenolics may also be secreted extracellularly as resins or cell wall material (Zucker, 1983). Breeders have sometimes increased the levels of trypsin inhibitors to produce insect-resistant varieties (Bressani, 1985). Gatehouse and Bouter (1983) found variation in the level of tannic acid and catechin from 10 IITA cowpea varieties. Singh *et al.* (1997) reported a positive relationship between resistance/susceptibility characteristics against aphids and qualitative and/or quantitative flavonoid content. Tannins are a major group of polyphenols which, when they occur in high quantities, are able to reduce tissue digestibility and palatability to herbivores and protect plants against microbial infection (Harborne and Turner, 1984). Phenols and their derivatives, mainly tannins are responsible for the colour of cowpea seeds but the level of the compound and its location in the different tissues are variable (Werker, 1997). The anthocyanin pigmentation of seed present in almost all cowpea varieties can be relatively high but varies with the geographic location (Bhatt, 1971). Other polyphenols, such as anthocyanin, contribute mainly to plant colours such as pink, red, mauve and shades of blue, and flavanoids have been implicated as ultraviolet protective agents in leaves (Harborne and Turner, 1984).

Plant tissues that are able to withstand the action of oxalic acid and cell-wall degrading enzymes (primarily endopolygalacturonase) may be resistant to hyphal invasion and infection by *S. rolfisii* (Punja *et al.*, 1985). These may include tissues that are heavily lignified or suberised, tissues in which an impermeable layer of phellogen is formed, tissues containing higher level of calcium (Bateman and Beer 1965; Punja *et al.*, 1985), phenolic compounds or oxalic acid oxidase (Franceschi and Horner, 1980), and tissues containing protein inhibitors of endopolygalacturonase (Punja, 1985). Initial demonstrations that phenols are significant components of the host response involved isolation and identification of phenols from tissues following inoculation (Kosuge, 1969). Improved technologies, such as high performance liquid chromatography (HPLC) help to isolation and identification of small amounts of phenols (Nicholson and Hammerschmidt, 1992). In cowpea, information is needed about the identification and

localisation of phenolic compounds, which may be involved in resistance or susceptibility of cowpea cultivars to damping-off and stem rot.

Screening is one of the important processes involved in breeding programmes of dry beans (Watt *et al.*, 1985). Resistant cultivars can be identified through screening methods and sources of resistance are incorporated into cultivars intended for improved production (Singh *et al.*, 1997). The screened cultivars could exhibit increased resistance to a wide range of diseases and insects, better tolerance to environmental stress, increased nitrogen-fixing capacity, better seed quality and improved efficiency in the limited soil nutrient utilisation (Watt *et al.*, 1985). Cowpea improvement programmes in Africa have received attention from 1970 at the International Institute of Tropical Agriculture (IITA) (Singh and Ntare, 1985). IITA developed reliable and fast screening techniques for studies and evaluation of *Striga* and *Alectra* segregating materials (Singh *et al.*, 1997). These screening methods include greenhouse and field screenings. The greenhouse screening uses pot culture techniques testing the field screening results (Singh *et al.*, 1997). However, these screening methods are still time consuming. Since cowpea is mostly utilised as food by small-scale farmers in many countries, a quicker method of screening cultivars before being dispersed is necessary. Furthermore, there is no screening method for resistance of cowpea cultivars to *S. rolfsii* damping-off and stem rot.

#### 2.10.3 Chemical control

Chemically, the diseases can be controlled by fertilising with an ammonium-type fertiliser, calcium compounds and, in some cases, by applying fungicides such as pentachloronitrobenzene (PCNB), captafol, and dichloran to the soil before planting or in the furrow during planting (Punja, 1985). *Sclerotium* rots are also controlled by chloroneb and prothiocarb, which are used as a soil drench or as an in-furrow spray near the roots of crops (Jones, 1987). Despite the effectiveness of synthetic fungicides, they have the drawbacks such as potential harmful effects on non-target organisms and environment, development of resistant races of pathogens, and possible carcinogenicity of some chemicals (Zaki *et al.*, 1998).

#### 2.10.4 Biological control and use of plant-derived fungicides

*Trichoderma* spp. successfully controlled *S. rolfsii* both in culture and in the greenhouse (Singh *et al.*, 1997). However, the control of the *Sclerotium* diseases using *Trichoderma*

in the field in north Brazil was found to be difficult, because the pathogen has a wide host range and can survive in the soil for a long time (Singh and Rachie, 1985).

Fungicidal effects of neem (*Azadirachta indica* A. Juss), papaya (*Carica papaya* L.) (Stoll, 1988; PEDUNE-BENIN, unpublished). and *Moringa oleifera* L. leaf and/or seed extracts were tested on the expression of diseases (Stoll, 1988; SIBAT, 1993). Seeds were soaked in the extract solution for 5 to 10 min and sowed. Results indicated that extracts reduced the expression of the diseases compared to non-treated seeds (PEDUNE-BENIN, unpublished).

#### 2.10.5 Integrated pest-disease management (IPM)

The IPM strategy includes the combination of more than one control measures: Use of resistant cultivars, agronomic practices, fungicides (synthetic with low dose and plant-based fungicides) and biological control (Singh *et al.*, 1997). The agronomic practices reduce the primary *S. rolfsii* inoculum in the field (Singh *et al.* 1997). The use of resistant cultivars limits the development of *S. rolfsii* diseases, which can be finally controlled by the use of an appropriate formulation of a biological control agent (such as *T. harzianum* or *Bacillus* sp.) and a low-dose fungicide.

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## **CHAPTER 3**

# **OCCURRENCE AND DISTRIBUTION OF COWPEA DAMPING-OFF AND STEM ROT AND ASSOCIATED FUNGI IN BENIN**

**OCCURRENCE AND DISTRIBUTION OF COWPEA DAMPING-OFF AND  
STEM ROT AND ASSOCIATED FUNGI IN BENIN**

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*Submitted to Journal of Agricultural Science for publication as:*

Adandonon, A., Aveling, T.A.S & Tamo, M. 2004. Occurrence and distribution of cowpea damping-off and stem rot and associated fungi in Benin.

**Abstract**

Damping-off and stem rot of cowpea is an important soilborne disease worldwide. Cowpea fields were randomly chosen in each agro-ecological zone in Benin and surveyed in 2001 and 2002 to determine the occurrence of the diseases throughout the country. Diseased plants, prevailing environmental conditions and cowpea grower cultural practices were recorded and causal agents associated with the disease identified. Results indicated that damping-off and stem rot were distributed throughout Benin. The disease incidence was higher in the South and Centre zones than in the other zones in the country. Among factors influencing disease incidence, cultural practices such as sole crop and no-till systems appeared to be most important. Isolated fungi included *Sclerotium rolfsii*, *Fusarium* spp., *Pythium ultimum*, *Rhizoctonia solani*, *Phoma pomorum*, *Rhizopus* sp. and *Trichoderma harzianum*. None of the *Fusarium*, *T. harzianum* or *Rhizopus* sp. isolates were pathogenic in the greenhouse. *Pythium* sp., *R. solani* and *P. pomorum* were infrequently isolated and few isolates caused the disease symptoms in the greenhouse. However, this is the first report of *P. pomorum* causing damping-off and stem rot of cowpea in Benin. *Sclerotium rolfsii* was by far the most common species isolated from all the agro-ecological zones and all isolates were pathogenic on cowpea in the greenhouse. *Sclerotium rolfsii* was considered to be the main causal agent of cowpea damping-off and stem rot in the Republic of Benin due to its wide distribution, high incidence and predominance on plants with damping-off and stem rot symptoms.

Keywords: cowpea, damping-off and stem rot, incidence, soilborne fungi



### 3.1 Introduction

Cowpea (*Vigna unguiculata* [L.] Walp) yield is often low due to losses caused by insect pests and diseases, including damping-off and stem rot (Singh *et al.*, 1997). Damping-off and stem rot of cowpea has been reported in many countries (Singh *et al.*, 1997; Aveling and Adandonon, 2000). Several causal agents have been associated with damping-off and stem rot of cowpea (Singh *et al.*, 1997) and in Southern Africa the causal agents were identified as *Pythium ultimum* Trow (Aveling and Adandonon, 2000) and *Rhizoctonia solani* Kühn (Adandonon, 2000). The diseases have also been reported on cowpea in Nigeria, Asia and Brazil, and are caused by fungi such as *Pythium aphanidermatum* (Edson) Fitzp., *R. solani*, *Phytophthora* sp., *Fusarium solani* (Mart.) App. and Wol. and *Sclerotium rolfsii* Sacc. (Singh *et al.*, 1997).

Outbreaks of disease are often triggered by specific environmental events that can occur before or during initial infection (Pieczarka and Abawi 1978; Wakeham *et al.*, 1997). Pieczarka and Abawi (1978) and Wakeham *et al.* (1997) reported that when environmental conditions prevailing in both air and soil are suitable, disease development might be greatly affected and colonisation of host tissues and disease spread can be rapid. Moreover, inappropriate cultural practices may lead to severe disease expression (Singh and Rachie, 1985). In Benin, the cultural practices vary from one cowpea production area to another and farmers prepare soil differently before sowing (PEDUNE-BENIN, 1995).

In the Ouémé valley, Benin, damping-off and stem rot is a major constraint to the production of cowpea (PEDUNE-BENIN, 1995) and the diseases were reported to be caused by *S. rolfsii* (Adandonon, 2000; Kossou *et al.*, 2001). However, no information exists on the countrywide distribution of the diseases, and the causal agents in each zone have yet to be identified. The main objectives of this study were to survey cowpea production-areas throughout the different agro-ecological zones to determine the distribution of the diseases in Benin and identify the associated soilborne fungal pathogens and their relative importance in cowpea fields in the country.

### 3.2 Materials and Methods

#### 3.2.1 Distribution and incidence of damping-off and stem rot in Benin

Surveys were conducted throughout the Republic of Benin, a West African Country bordered in the north by Niger and Burkina Faso, in the south by the Atlantic Ocean, in the east by the Federal Republic of Nigeria and in the west by Togo Republic. The

country consists of five agro-ecological zones, namely South, Centre, South Borgou/South Atacora, North Borgou and Atacora (Fig. 3.1) (INRAB, 1995). The surveys were done in the farmers' cowpea fields between 2001 and 2002 seasons. In each agro-ecological zone, 30 cowpea fields chosen at random were surveyed, at seedling, flowering and podding stages for the occurrence of damping-off and stem rot. In each field, six 2-m<sup>2</sup> plots were marked and all cowpea plants showing symptoms of the diseases were recorded. Two to four diseased plants were randomly selected, shoots were excised and hypocotyls or stems were collected for fungal identification in the laboratory.

### 3.2.2 Field evaluations of the effects of environmental conditions and cowpea cropping systems on disease incidence

Rainfall was recorded daily using a funnel-type gauge positioned 1.5 m above the soil surface in the field. The air temperature and relative humidity were measured daily in the field for the duration of the experiment using a hygrothermograph placed at a height of 30 cm above the cowpea plant canopy. To ensure measurement accuracy, the instrument was housed in a Stevenson's screen and protected from direct sunlight and rain. Cropping systems, such as sole cropping and no-till practices were recorded.

### 3.2.3 Fungal identification and pathogenicity

The diseased samples were surface-sterilised, plated onto potato dextrose agar (PDA) and incubated at  $27 \pm 1$  °C. Plates were examined three to 15 d later for identification of fungi associated with the symptoms observed (Domsch *et al.*, 1980). Pathogenicity of each isolate was tested in the greenhouse at the International Institute of Tropical Agriculture (IITA), Cotonou Station, Benin. Soil inoculation with each isolated species was done by means of the millet (*Panicum miliaceum* L.) seed inoculum technique (Weideman and Wehner, 1993). Five 5 mm diam. discs cut from the actively growing edge of *S. rolfsii* colony on PDA were used to inoculate 50 g of the millet seeds. Prior to the inoculation, the millet seeds were steeped in water for 48 h and autoclaved for 45 min at 120°C. The inoculated-millet seeds were incubated for 21 d at 27 °C, and then used to inoculate sandy loam soil. The soil was pasteurised by aerated steam (60 °C for 60 min) and stored for 21 d before inoculation with the milled seed inoculum (Pieczarka and Abawi, 1978). The soil inoculation was done by mixing 17 g of the inoculated-millet seeds with 1 kg pasteurised sandy loam soil. The control treatments consisted of

pasteurised soil to which 17 g sterile millet seed was added and pasteurised soil without millet seed. Seeds of a local cowpea cultivar (Tchawé kpayo) susceptible to damping-off and stem rot (Adandonon, 2000) were surface disinfected in 1 % NaOCl for 2 min, rinsed twice in sterile distilled water (SDW) and then planted in pots (14 cm diam. x 18 cm height) using four seeds per pot filled with inoculated soil. Pots were kept at 21-30 °C in the greenhouse, watered every 2 d and the number of seedlings showing symptoms of damping-off and stem rot in each pot were recorded at seven and 30 d after planting.

To fulfil Koch's postulates, dying seedlings and plants were removed at each observation and plated onto PDA media. A fungal species was considered to be pathogenic when its inoculation to cowpea seedlings, using milled seed inoculum technique, in the greenhouse resulted in at least one diseased seedling or plant with the typical symptoms of the diseases. The reisolated fungus was cultured on PDA and colony characteristics were recorded and compared to the original isolates.

#### 3.2.4 Statistical analysis

The percentage infected plants in field was arcsine ( $Y/2$ ) transformed. Analysis of variance was performed using the general linear model (GLM) procedure in the SAS System (SAS, 1997) and mean separations were done using the Student Newman Keuls (SNK) option. Correlation matrix and multiple regression analyses were performed, taking into account all variables of the system. To avoid overestimating the variable impact, the multiple coefficient of determination was adjusted and computed as follows (Anderson *et al.*, 2003):

$$R_a^2 = 1 - (1 - R^2)(n - 1)/(n - p - 1) \text{ where}$$

$R_a^2$  = adjusted multiple coefficient of determination

$R^2$  = multiple coefficient of determination

n = number of observation

p = number of independent variables

### 3.3 Results

#### 3.3.1 Distribution and incidence of the damping-off and stem rot in Benin

Seedling damping-off symptoms observed in the field included necrotic, water-soaked and brown discoloured lesions on the hypocotyls (Fig. 3.2). The diseased seedlings wilted and fell over onto the soil surface. Stem rot was observed on mature cowpea

plants, which showed whitish or brown lesions at the base of the stem and wilting, but remained upright. The lesions on these plants were covered with white mycelium in which brown sclerotia were often embedded. The disease symptoms were found in all five agro-ecological zones (Table 3.1) in Benin. The incidence of the diseases varied from zone to zone and the highest incidence was recorded in the South and Centre zones of Benin (Table 3.1).

### 3.3.2 Field evaluations of the effects of environmental conditions and cowpea cropping system on disease incidence

Results (Table 3.1) show that cowpea is cultivated mainly as sole crop (field with one crop species) in the South and the percentage of cowpea fields under sole cropping decreases to the North of the country. The percentage of fields with no-till was highest in the South and the values became progressively lower toward the North of the country where the lowest percentage was recorded. However, the recorded rainfall did not follow the same pattern. The rainfall was highest in the South followed by Atacora, and North Borgou recorded the lowest rainfall during this experiment (Table 3.1). There was a positive correlation between disease incidence and recorded minimum temperature, air humidity, rainfall, sole crop and no-till, as shown in the correlation matrix (Table 3.2). The estimated multiple regression equation, including all measured parameters of the system was significant ( $P < 0.05$ ) with an estimated coefficient of determination  $R^2 = 0.987$ . However, all the independent variables, except no-till and sole crop, had  $p$ -values greater than 0.05. In the final step of the backward elimination procedure, only no-till and sole crop had  $p$ -values that were less than 0.05. The estimated multiple regression equation,  $y = 1.295 + 0.0707x_1 + 0.0269x_2$ , was significant ( $P < 0.05$ ) with an adjusted coefficient of determination,  $R^2 = 0.995$ , indicating a good fit of the data.

### 3.3.3 Fungal identification and pathogenicity

Soilborne fungi isolated from diseased seedlings or plants included *Sclerotium rolfsii*, *Fusarium* spp., *Phoma pomorum* Thüm, *Pythium* sp., *Rhizoctonia solani*, *Rhizopus* sp., and *Trichoderma harzianum* (Table 3.3). *Sclerotium rolfsii* was the predominant fungus isolated (isolation frequency of 98 - 100 %) from samples from all the agro-ecological zones. A culture was deposited in the National Collection of Fungi, Biosystematics Division, Plant Protection Research Institute, Pretoria (PREM 57252). In all pathogenicity tests, soil inoculation with *S. rolfsii* alone always resulted in disease

symptoms. None of the isolates of *Fusarium* spp, *T. harzianum* or *Rhizopus* sp. caused disease (Table 3.3). *Pythium* sp. and *R. solani* were isolated only from South Borgou/South Atacora and *P. pomorum* only from Northern Borgou with isolation frequencies of 5.03 %, 3.14 % and 8.16 %, respectively (Table 3.3). Only two of the eight *Pythium* spp., one of the five *R. solani* and one of the four *P. pomorum* isolates caused disease symptoms in the greenhouse.

### 3.4 Discussion

The occurrence and distribution of a specific disease varies from one place to another, but some diseases occur in and cause significant damage across many regions of the world (Emechebe and Florini, 1997; Singh *et al.*, 1997). Damping-off and stem rot has been reported on cowpea on most of the continents including West and Central Africa (Onuorah, 1973), Tropical Asia (Singh and Rachie, 1985), Latin America (Brazil, Nicaragua) (Singh and Allen, 1979; Singh and Rachie, 1985) and the United States of America (USA) (Singh and Rachie, 1985). The fact that damping-off and stem rot symptoms were found in the present study in all five agro-ecological zones of Benin emphasises the importance and widespread distribution of the diseases in the country. This is the first report of the countrywide distribution and incidence of damping-off and stem rot of cowpea in Benin. However, disease incidence was uneven, indicating that the environmental conditions favourable for the diseases varied over the five agro-ecological zones. The disease incidence was the highest in the South and decreased to the North, with the lowest disease incidence recorded in the two northern agro-ecological zones of the country (North Borgou and Atacora).

The recorded environmental data showed that the South received the most rain and had the highest humidity (INRAB, 1995). These conditions are said to be influenced by the presence of many lakes and lagoons and the proximity of the Atlantic Ocean (INRAB, 1995). This zone, in terms of rainfall and air humidity, is followed by Atacora, South Borgou/South Atacora, Centre and North Borgou, respectively, in decreasing order. The Atacora zone is characterised by the presence of a mountain range, called Atacora, whereas South Borgou/South Atacora has the largest forest zone in Benin, which may influence the rainfall in this zone (INRAB, 1995). In general, the cultural practices varied from one cowpea production area to another and farmers prepared soil differently before sowing (PEDUNE-BENIN, 1995). Farmers in the South and Centre quite often practiced no-tillage coupled with sole cropping. In the no-till system, plants

are directly exposed to the existing initial inoculum of the pathogen and therefore disease is initiated quite rapidly (Singh and Rachie, 1995). The other farmers of these zones, particularly the Centre zone, practice a till system, then burying weeds and remaining infected plants. The burial of the infected plant favours the spread of soilborne fungal diseases (Dekker, 2003) and might also be one of the factors explaining the level of the disease incidence observed in the Centre zone. Furthermore, the sole crop system, consisting of one crop species, causes the pathogen to easily spread to nearby plants. As a result the sole crop coupled with the no-till system leads to a high disease incidence and severe epidemic conditions (Coakley, 1988). In contrast, in the South Borgou/South Atacora, North Borgou and Atacora zones, the cropping system consisted of burning the infected plant residues before ploughing (tilling) the soil. This cropping, coupled with the mixed crop system, often leads to the elimination or drastic reduction of initial inoculum of soilborne pathogens (Sumner *et al.*, 1981) and therefore to low disease incidence (Singh *et al.*, 1997).

Several early reports have shown that in an intercropping or mixed crop system, the populations of several pests and disease incidence are reduced and yields increased accordingly (Singh *et al.*, 1997; Coakley, 1988). Coakley (1988) and Huber and Gillespie (1992) indicated that when large acreages are planted to the same varieties of crop, a greater likelihood exists that infection will result in a high disease incidence and even in an epidemic. In contrast, the disease develops rather slowly in an intercropping system where plants of different species are intermingled (Singh and Rachie, 1985; Coakley, 1988). In the present study, no-till and sole crop factors positively correlated with disease incidence with a good fit for the estimated regression analysis. These two factors might, therefore, explain why the disease incidence was higher in the South and Centre zones, and lower in the three northern zones, especially North Borgou and Atacora, although the amount of rainfall recorded in the Atacora zone was relatively important.

Among the isolated fungi in the present study, *S. rolfsii* was the most predominant in all the five agro-ecological zones and all isolates were pathogenic in the greenhouse. *Sclerotium rolfsii* was reported not to prevail in cool temperate zones, but rather in warm climates (Punja, 1985). The fungus is a destructive soilborne pathogen and causes severe damage in the Tropics and Subtropics (Punja, 1985; Mukherjee and Raghu, 1997). The Republic of Benin falls into the Sudan Savannah agro-ecological zone like most West African countries (Singh and Rachie, 1985) and prevailing conditions are

among those favourable for the development of damping-off and stem rot caused by *S. rolfsii* (Punja, 1985). The five agro-ecological zones of the country are characterised by annual temperatures ranging from 26 to 35 °C (INRAB, 1995), which permit the growth of the pathogen (Punja, 1985). The cowpea cropping soils in the South and Centre are mostly ferruginous with clayish in the valley lowlands where cowpea is also cultivated after natural flooding (INRAB, 1995). The types of soils in the remaining three agro-ecological zones are similar to those in the South and Centre zones, except for the presence of stony or rocky barren soils, mainly in Atacora (INRAB, 1995). The climate and soil conditions in the South and Centre zones might be more favourable for the development of the fungus than those in North Borgou where the air humidity is low, sometimes only 32 %, and maximum temperatures often reach 40 °C, or those in Atacora with unfertile stony soils coupled with till and mixed cropping systems (INRAB, 1995). These results correspond to early reports on the ecology of *S. rolfsii* (Punja, 1985; Mukherjee and Raghu, 1997; Singh *et al.*, 1997). In the current work, other isolated fungi included *Fusarium* spp., *Rhizopus* sp., *Trichoderma harzianum*, *Pythium* sp., *R. solani* and *P. pomorum*. None of the *Fusarium*, *Rhizopus* sp. or *T. harzianum* isolates were pathogenic in the greenhouse, indicating these fungal species isolated in the present study might be considered non-pathogenic. Because *Pythium* sp., *R. solani* and *P. pomorum* were infrequently isolated during the survey and few isolates caused disease symptoms in the greenhouse, these fungi might be regarded as minor causal agents of the diseases in Benin. However, this is the first report of *P. pomorum* causing damping-off and stem rot of cowpea in Benin. One pure culture of *P. pomorum* was deposited in the Collection of Fungi, Plant Health Management Division, International Institute of Tropical Agriculture (IITA), Cotonou, Benin (IITA 630).

Damping-off and stem rot of cowpea and associated causal agents were reported in earlier studies (Punja, 1995; Singh *et al.*, 1997; Aveling and Adandonon, 2000). However, the extent to which the diseases occur in Benin was not known. This current study provides the first documentation on the occurrence and distribution of damping-off and stem rot and the associated causal fungi in the Republic of Benin. Furthermore, the study adds information on the distribution of the diseases worldwide. Damping-off and stem rot is distributed countrywide in Benin. Pending an appropriate and sustainable solution to the diseases, control measures could include the gathering and burning of infected plants, where applicable, and planting seeds on raised beds (till) with appropriate plant spacing, as suggested by Lucas *et al.* (1993).



### 3.5 Acknowledgments

Research was financed by the International Institute of Tropical Agriculture (IITA), Nigeria. Thanks are due to Dr Y. D. Arodokoun, INRAB, Cotonou, Dr J. van der Waals, University of Pretoria, Pretoria and Mrs J. Hotegni, IITA-Cotonou.

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**Table 3.1**

Distribution, incidence of damping-off and stem rot of cowpea, cropping system and rainfall recorded per agro-ecological zone throughout Benin in 2001-2002

Ecological zones <sup>m</sup>	Disease incidence (%)	Rainfall (mm)	Sole crop <sup>r</sup> (%)	No-till <sup>n</sup> (%)
South	8.1 <sup>l</sup> ± 0.65 c	1378.3 ± 10.61 d	93.2 ± 2.43 d	85.2 ± 1.87 c
Centre	7.1 ± 0.54 bc	1091.8 ± 9.56 b	77.5 ± 1.85 c	72.3 ± 1.58 c
S Borgou/S Atacora	5.7 ± 0.49 b	1105.0 ± 9.87 b	35.6 ± 2.21 b	53.4 ± 1.83 b
North Borgou	2.3 ± 0.57a	913.6 ± 10.32 a	20.3 ± 2.08 ab	16 ± 1.85 a
Atacora	2.2 ± 0.60 a	1174.4 ± 10.43 c	6.4 ± 1.92 a	8.7 ± 1.87 a

<sup>m</sup>Agro-ecological zones: S Borgou/S Atacora = South Borgou/South Atacora lzone. <sup>l</sup>Values are followed by the standard error. Within a column, values not followed by the same letters are significantly different ( $P < 0.05$ ) according to Student Newman Keuls. Degree of freedom for the error term: 19. <sup>r</sup>Sole crop (%) = Percentage of fields planted with cowpea as a sole crop. <sup>n</sup>No-till (%) = percentage of cowpea fields without tillage.

**Table 3.2**

Correlation matrix of parameters measured during the cowpea damping-off and stem rot survey in Benin in 2001-2002

Parameters <sup>z</sup>	TMin.	TMax.	HMin.	HMax.	Rainy days	Rainfall	Sole crop	No-till	Diseased plants (%)
TMin.	1								
TMax.	-0.035	1							
HMin.	-0.920	0.077	1						
HMax.	-0.924	0.120	0.963	1					
Rainfall	-0.269	0.057	0.519	0.412	1				
Rainy days	-0.484	0.445	0.623	0.524	0.625	1			
Sole crop	-0.865	0.049	0.943	0.966	0.504	0.500	1		
No-till	-0.945	0.081	0.945	0.984	0.344	0.502	0.962	1	
Diseased plants (%)	-0.939*	0.124	0.936*	0.979*	0.357	0.520*	0.945*	0.990*	1

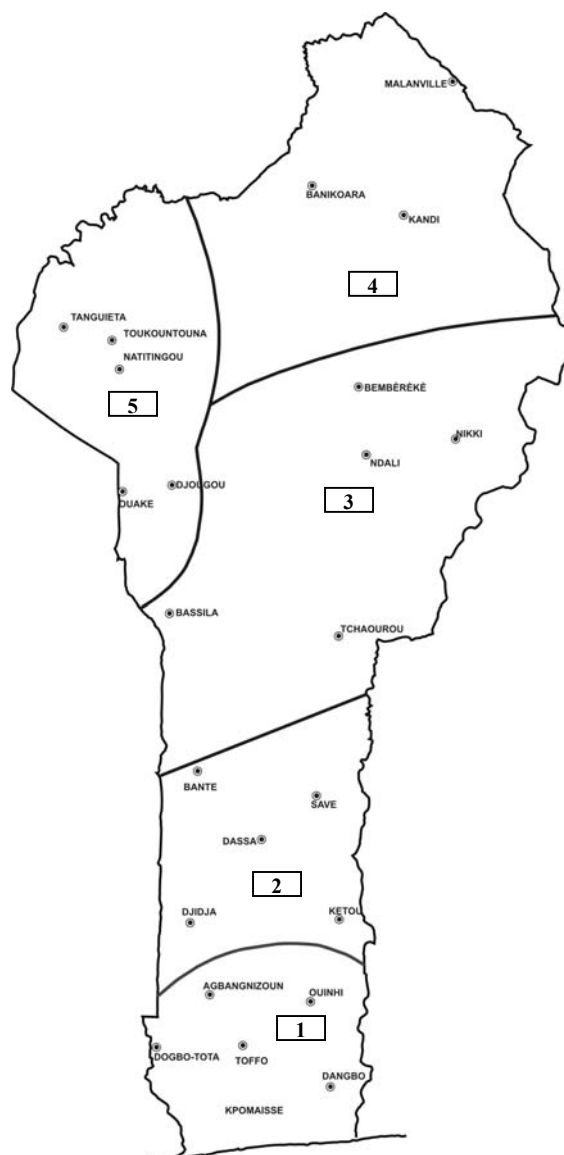
<sup>z</sup>TMin. = Minimum temperature; TMax. = Maximum temperature; HMin. = Minimum humidity; HMax. = Maximum humidity. Sole crop refers to fields planted with cowpea as a sole crop. "\*" indicate the correlation coefficient is significant.

**Table 3.3**

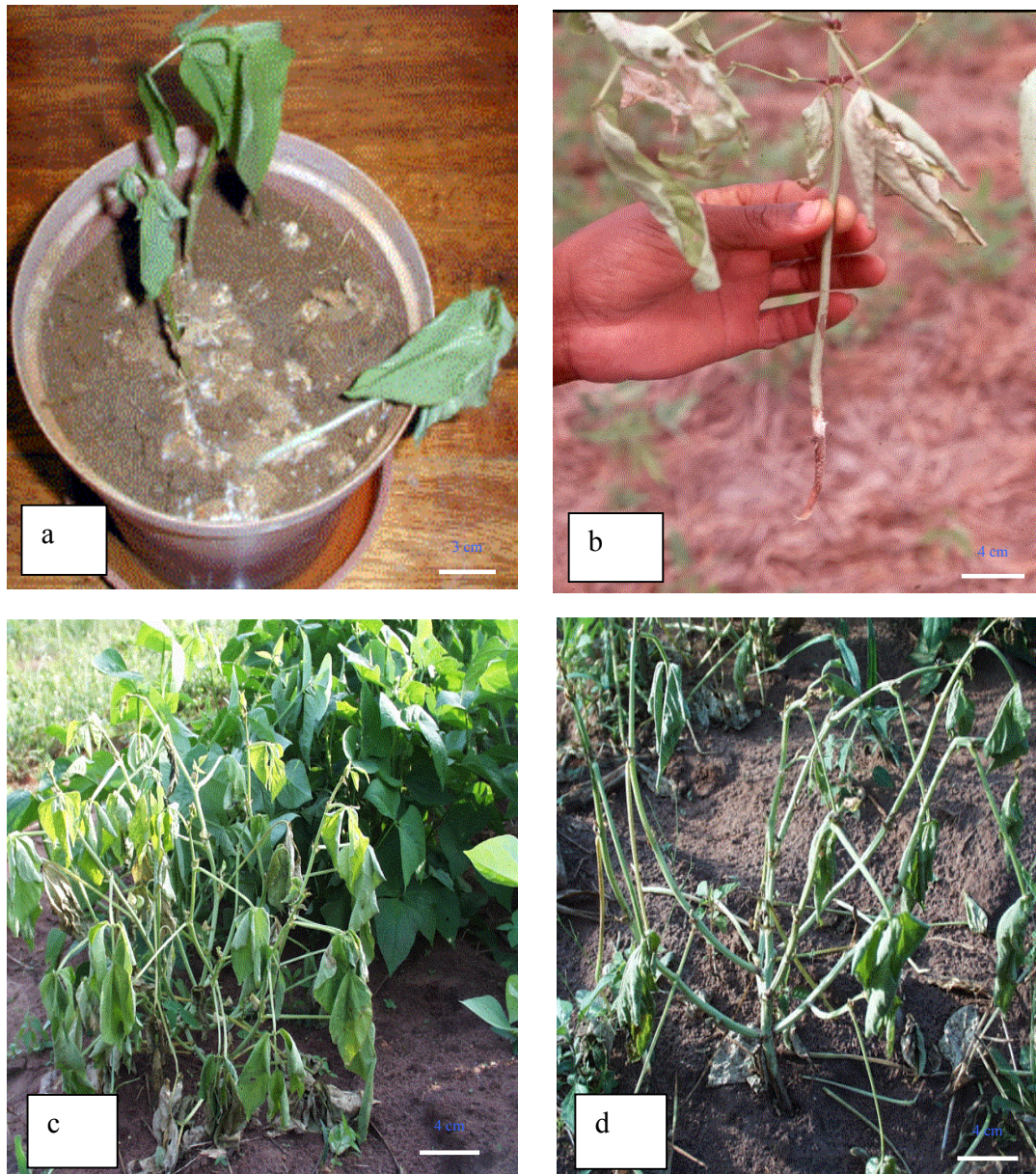
Isolation frequencies and *in vivo* pathogenicity of the fungi isolated from cowpea damping-off and stem rot cowpea seedlings or plants per agro-ecological zone throughout Benin in 2001-2002

Fungi	Parameters <sup>m</sup>	Ecological zones <sup>1</sup>				
		South	Center	South Borgou- South Atacora	North Borgou	Atacora
<i>Fusarium</i> spp.	Iso. freq. (%)	27.1 <sup>r</sup> ± 2.14 d	23.1 ± 1.93 cd	11.3 ± 1.84 a	20.4 ± 1.92 bc	18.9 ± 1.57 b
	Pathogen.	(45) 0	(46) 0	(18) 0	(10) 0	(4) 0
<i>P. pomorum</i>	Iso. freq. (%)	0 ± 0 a	0 ± 0 a	0 ± 0 a	8.2 ± 0.68 b	0 ± 0 a
	Pathogen.	(0) 0	(0) 0	(0) 0	(4) 1	(0) 0
<i>Pythium</i> sp.	Iso. freq. (%)	0 ± 0 a	0 ± 0 a	5.0 ± 0.21 b	0 ± 0 a	0 ± 0 a
	Pathogen.	(0) 0	(0) 0	(8) 2	(0) 0	(0) 0
<i>R. solani</i>	Iso. freq. (%)	0 ± 0 a	0 ± 0 a	3.1 ± 0.12 b	0 ± 0 a	0 ± 0 a
	Pathogen.	(0) 0	(0) 0	(5) 1	(0) 0	(0) 0
<i>Rhizopus</i> sp.	Iso. freq. (%)	1.8 ± 0.04 b	0 ± 0 a	3.8 ± 0.07 c	0 ± 0 a	0 ± 0 a
	Pathogen.	(3) 0	(0) 0	(6) 0	(0) 0	(0) 0
<i>S. rolfsii</i>	Iso. freq. (%)	100 ± 0.45 a	100 ± 0.69 a	98.7 ± 0.87 a	100 ± 0.38 a	100 ± 0.33 a
	Pathogen.	(80) 80	(80) 80	(80) 80	(49) 49	(22) 22
<i>T. harzianum</i>	Iso. freq. (%)	12.1 ± 0.94 c	5.0 ± 0.54 b	4.4 ± 0.34 b	0 ± 0 a	4.5 ± 0.55 b
	Pathogen.	(20) 0	(10) 0	(7) 0	0 (0)	1 (0)

<sup>m</sup>Iso. freq. (%) = Frequency of fungal isolation from diseased plants. Isolation frequency (%) of a fungal species for a given zone is the number of diseased plants with the fungal species divided by the total number of diseased plants collected from the specific agro-ecological zone multiplied by 100. Pathogen. = Pathogenicity; this is the number of isolates causing damping-off or stem rot symptoms on cowpea (in parenthesis) out of the total number of isolates tested (not in parenthesis). <sup>1</sup>Agro-ecological zones: studied zones. <sup>r</sup>Values are followed by the standard error. Within a column, values not followed by the same letters are significantly different ( $P < 0.05$ ) according to Student Newman-Keuls. Degree of freedom for the error term: 19.



**Figure 3.1** Map of Benin with its five agro-ecological zones and the different regions surveyed for the occurrence and distribution of damping-off and stem rot of cowpea. 1 = South; 2 = Centre; 3 = South Borgou/South Atacora, 4 = North Borgou and 5 = Atacora (INRAB, 1995). Dots are surveyed regions.



**Figure 3.2** Cowpea damping-off seedlings and mature plants with stem rot diseases. a-: Damping-off seedling falls over on the ground; b-: Mature plant affected by stem rot show whitish or brown lesions of the base of the stem; c-d-: Mature plants with stem rot remain upright but become yellow and wilt.



## **CHAPTER 4**

# **ETIOLOGY OF AND EFFECT OF ENVIRONMENTAL FACTORS ON DAMPING-OFF AND STEM ROT OF COWPEA IN OUÉMÉ, BENIN**

## CHAPTER 4

### ETIOLOGY OF AND EFFECT OF ENVIRONMENTAL FACTORS ON DAMPING-OFF AND STEM ROT OF COWPEA IN OUÉMÉ, BENIN

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*Submitted to Phytoparasitica for publication as:*

Adandonon, A., Aveling, T. A. S., Labuschagne, N. and Ahohuendo, B. C. 2004. Etiology of and effect of environmental factors on damping-off and stem rot of cowpea in Benin.

#### **Abstract**

Damping-off and stem rot are two disease syndromes of cowpeas in the Ouémé valley, Benin. Of the fungal species isolated from diseased plants in the field during a two-year experiment (2001 and 2002), *Sclerotium rolfsii* was found to be solely responsible for these two diseases. In a fungal interaction test in the greenhouse, *Trichoderma harzianum* suppressed the growth of *S. rolfsii* resulting in the absence of diseases. Measurement of *S. rolfsii* initial inoculum, disease incidence and soil moisture in cowpea field plots revealed a positive correlation ( $r=+0.71$ ) between these parameters. The disease incidence decreased with increase in distance from the river (area of high soil moisture and high inoculum). Mean air temperature and relative humidity were relatively constant during the initial stage of cowpea growth, during the two-year experiment in the field. This is the first comprehensive study of the effects of environmental factors on the incidence of cowpea damping-off and stem rot caused by *S. rolfsii* in Benin.

Keywords: initial inoculum, soil moisture, susceptibility, temperature, *Vigna unguiculata*



#### 4.1 Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is an important grain legume in West Africa (Singh and Rachie, 1985) and provides an inexpensive source of protein for the poor urban and rural population (Alghali, 1991). Unfortunately, cowpea yield is low and among the causes is its susceptibility to many diseases (Aveling and Adandonon, 2000) such as damping-off and stem rot. These diseases have been reported in many countries in tropical Asia, USA, Nigeria (Singh and Rachie, 1985) and South Africa (Adandonon, 2000; Aveling and Adandonon, 2000). Damping-off and stem rot diseases are caused by many different species of fungi (Aveling and Adandonon, 2000; Emechebe, 1981; Singh and Rachie, 1985) that might act individually or through an interaction in a fungal complex (Pieczarka and Abawi, 1978). Interactions among soilborne plant pathogens have been demonstrated to greatly influence disease incidence and severity on many crops (Powell, 1971). Moreover, outbreaks of disease are often triggered by specific environmental events that can occur long after initial infection (Pieczarka and Abawi, 1978; Wakeham *et al.*, 1997). Pieczarka and Abawi (1978) and Wakeham *et al.* (1997) reported that when environmental conditions prevailing in both air and soil are suitable, disease development might be greatly affected and colonisation of host tissues and disease spread can be rapid.

A recent survey conducted in Benin (Chapter 3) indicated that the disease is distributed throughout the country. The incidence of the disease varies and also appeared to be dependent on the environmental conditions prevailing in a specific zone. In the Ouémé valley, located in the South zone of Benin, cowpea is a major cash crop for growers. Unfortunately, damping-off and stem rot of cowpea are the most important disease syndromes of the crop during the production season, which starts after natural flooding. Damping-off and stem rot were reported in the valley (PEDUNE-BENIN, 1995) and were said to result in yield losses ranging from 19 to 40 % (PEDUNE-BENIN, 1995). During a recent survey (Chapter 3), the highest disease incidence was recorded in this particular valley. Further information is required about the effect of environmental conditions on the severity of the diseases in this area. The objectives of the present study were, therefore, to study aspects of the epidemiology of the diseases in the Ouémé valley including the influence of initial inoculum, rainfall, air temperature, relative humidity, and soil moisture on the incidence of damping-off and stem rot of cowpea, and secondly, to investigate the effect of interactions in the fungal complex responsible for the disease in the Ouémé valley.

## 4.2 Materials and Methods

### 4.2.1 Collection of plant material

Surveys were conducted in farmers' fields in six villages in the Ouémé valley (Agbonou, Agonguey, Agonlin, Dannou, Gangban and Wozounme) during the 2001 and 2002 cowpea planting seasons. Three bands, each representing a block and running perpendicular to the river, were demarcated, about 150 m apart. Five fields at 200 m-intervals were located along each band. The fields were planted with Tchawé kpayo, the local cowpea cultivar, cultivated and weeded by farmers using traditional cultural practices. In each field, a plot of 10 x 20 m was marked, within which six 2 m<sup>2</sup> sub-plots were demarcated. All cowpea plants within each sub-plot were examined and the number of plants showing symptoms of damping-off or stem rot were recorded and tape marked at seven, 15 and 30 d after planting. Disease assessment of these two disease syndromes was expressed as disease incidence. Two plants with the disease symptoms were randomly selected and dug from each 2 m<sup>2</sup> sub-plots. Shoots were excised and hypocotyls or stems were collected and transported to the laboratory for fungal pathogen isolation and identification.

### 4.2.2 Isolations

Diseased stem tissue was cut into small segments ( $\pm 2 \text{ mm}^2$ ), surface disinfected with 0.5 % sodium hypochlorite (NaOCl) for 15 s, rinsed twice in SDW, blotted dry on sterile tissue paper and plated onto potato dextrose agar (PDA) amended with 0.025 % chloramphenicol. After 2 d' incubation at 25 $\pm$ 1 °C, pure cultures were obtained by transferring fungal colonies to new PDA and water agar (WA) plates and incubating at 25 $\pm$ 1 °C under fluorescent light for five to 10 d. The pure fungal cultures were maintained on PDA slants at 4 °C.

### 4.2.3 Identification of fungal isolates

#### *Fungal colony morphological characteristics*

The radial growth of the colonies was measured after 3 d incubation, and the colony colour and number of produced sclerotia were recorded. Isolate identification was done using the Mycology Guidebook (Stevens, 1974) based on colony morphology, growth, spores, conidia and sclerotia size and shape. The system of Nelson *et al.* (1983) was also followed for the identification of some of the fungi whereas the keys of Domsch *et al.* (1980) were used for the identification of others. One pure culture was deposited in

the National Collection of Fungi, Biosystematics Division, Plant Protection Research Institute, Pretoria (PREM 57252) and the remainder in the Collection of Fungi, Plant Health Management Division, International Institute of Tropical Agriculture, Cotonou, Benin (isolate codes: IITA 407, IITA 408, IITA 409, IITA 410, IITA 411, IITA 412).

#### 4.2.4 Pathogenicity tests

Pathogenicity of each isolate was tested in the greenhouse at the International Institute of Tropical Agriculture (IITA), Cotonou Station, Republic of Benin. Soil inoculation was done as described earlier, in Chapter 3.

Cowpea seeds of the Tchawé kpayo cultivar were obtained from the Ouémé valley village. The seeds were surface disinfected in 1 % NaOCl for 2 min, rinsed twice in SDW and then planted in pots (14 cm diam. x 18 cm height) filled with isolate-inoculated soil. There were four seeds per pot, three pots per isolate and repeated four times. The pots were kept at 21-30 °C in the greenhouse, watered every 2 d and the number of seedlings showing symptoms of damping-off and stem rot in each pot were recorded at seven and 30 d after planting. Koch's postulates were fulfilled as described in Chapter 3.

#### 4.2.5 Effect of *in vivo* interactions among *F. oxysporum*, *F. scirpi*, *S. rolfsii* and *T. harzianum* on damping-off and stem rot of cowpea in the greenhouse

The *in vivo* interaction experiment was carried out in the greenhouse to test the synergistic or antagonistic effect of the different fungal species on cowpea using the technique of millet seed inoculum (Weideman and Wehner, 1993), described in Chapter 3. The experimental design was a randomised block design with four replicates of 19 treatments (Table 4.1). Tchawé kpayo cultivar seeds were surface disinfected and planted in pots as described earlier. The number of damping-off seedlings was recorded 3 d after planting and at 2-d intervals thereafter until 30 d after planting.

#### 4.2.6 Field evaluation of the effects of environmental conditions on disease development

On-farm trials were conducted during 2001 and 2002 in a village (Agonguey) in the Ouémé valley to determine the effect of environmental conditions (rainfall, air temperature, relative humidity and soil moisture) and natural field initial inoculum density on disease incidence in cowpea. The village is transected by a permanent river

that, each year, toward the end of the rainy season (August-September), overflows and floods the fields of the village. Cowpea is grown after the drainage of the water from the fields. The fields are known to have a history of damping-off and stem rot of cowpea (PEDUNE-BENIN, 1995). During the 2001 cowpea planting season experiment, three bands were demarcated, planted with Tchawé kpayo, and the cultural practices were as described earlier. The experimental design was a complete block with each band representing a block, replicated three times along the river. A 1.0 kg soil sample from a depth of 20 cm was taken at planting from each subplot for initial inoculum density determination. The wet-sieving method described by Punja *et al.* (1985) was used to directly recover and enumerate sclerotia from the soil samples. Soil moisture was measured gravimetrically from a 20 cm-deep soil sample, taken per subplot at planting and at 7-d intervals until harvest, using the technique of Nielsen *et al.* (1995). Seven d after planting and subsequently at 7-d intervals until harvest, all cowpea plants within each sub-plot were examined and the number of seedlings or plants showing symptoms of damping-off or stem rot recorded and tape marked to establish disease incidence. Rainfall was recorded daily using a funnel-type gauge positioned 1.5 m above the soil surface in the field. The air temperature and relative humidity were measured daily in the field for the duration of the experiment using a hygrothermograph placed at a height of 30 cm above the cowpea plant canopy. To ensure measurement accuracy, the instrument was housed in a Stevenson's screen and protected from direct sunlight and rain.

In the second experiment during the 2002 cowpea planting season, three cowpea cultivars Tchawé kpayo, Tchawé daxo and IT83D-326-2 were planted per block in the field. Tchawé kpayo is a susceptible cultivar and Tchawé daxo is a tolerant cultivar being grown by farmers in the valley whereas IT83D-326-2 is known to be moderately tolerant to damping-off and stem rot (PEDUNE-BENIN, 1995). The experimental fields were located along the bands demarcated along the river as described above and each cultivar was separately grown in a 10 x 20 m-plot (per field). The cultural practices, sampling and collection of environmental data were as described earlier.

#### 4.2.7 Statistical analyses

The statistical analysis was performed as described in Chapter 3.

### 4.3 Results

#### 4.3.1 Identification of fungal isolates

The fungal species and the incidence of the most commonly isolated species are given in Table 4.2. Four fungal species, *Fusarium oxysporum* Schlecht., *Fusarium scirpi* Lamb. and Faut., *Trichoderma harzianum* Rifai and *Sclerotium rolfsii* Sacc were isolated from plant samples collected from the field in 2001. Although all these species were present in all six villages, their prevalence was not consistent. In most cases, *S. rolfsii* was the only fungus isolated from the diseased plants. *S. rolfsii* and *F. oxysporum* were isolated from 2.91 % of the same plant samples, *S. rolfsii* and *F. scirpi* from 1.5 %, *S. rolfsii*, *T. harzianum* and *F. oxysporum* from 4.32 % and *S. rolfsii*, *F. scirpi* and *T. harzianum* from 0.5 %. Incidences of the fungal species showed a similar trend during the second field experiment.

#### 4.3.2 Pathogenicity tests

None of the *F. oxysporum*, *F. scirpi*, or *T. harzianum* isolates caused disease on cowpea in these experiments. Damping-off and stem rot occurred when cowpea seeds were planted in soil inoculated with *S. rolfsii* only (Table 4.2). Some plants showed stem infection but remained upright. Symptoms corresponded with those observed in the field and also with those described by Singh and Rachie (1985) on cowpea. *S. rolfsii* was re-isolated from the seedlings and produced cultures identical to those of the original isolate which was used as inoculum.

#### 4.3.3 Effect of *in vivo* interactions among *F. oxysporum*, *F. scirpi*, *S. rolfsii* and *T. harzianum* on damping-off and stem rot of cowpea in the greenhouse

When *S. rolfsii* isolates were paired with *F. oxysporum* or *F. scirpi* and incorporated into the soil, it resulted in significantly fewer diseased plants than when the *S. rolfsii* isolates were used on their own (Table 4.1). For example, the percentage of diseased plants was 75 % for the *S. rolfsii* isolate from Agonguey as single inoculum and 60.75 % when the isolate was combined with *F. oxysporum*. The trend was the same when *F. scirpi* was paired with the *S. rolfsii* isolates. However, when the *S. rolfsii* isolates were paired with *T. harzianum* or in any other combination including *T. harzianum* and incorporated into the soil, no cowpea damping-off or stem rot was recorded (Table 4.1).

#### 4.3.4 Field evaluation of the effects of environmental conditions on disease development

Damping-off symptoms observed in the fields included necrotic, water-soaked and brown lesions on the hypocotyl. The diseased seedlings showed wilting and fell over on the ground. When cowpea plants are affected at a later growth stage, the diseases are called stem rot. The affected plants developed the above mentioned symptoms, but remained upright. The lesions were covered with white mycelium in which brown sclerotia were embedded.

The initial inoculum density of *S. rolfsii* recorded in the field is shown in Table 4.3. The density was high closer to the river and the lowest in the plot farthest from the river (Table 4.3). A similar trend was observed for the two-year observation. Figure 4.1 shows the percentage diseased plants in the field. Disease incidence was high two weeks after planting and decreased thereafter. From five weeks after planting, no new damping-off seedlings or stem rot plants were recorded in the field. The incidence of damping-off and stem rot was higher near to the river than further away from it (Table 4.4), regardless of the cultivar, and positively correlated ( $r = +0.71$ ) with initial inoculum density. Soil moisture (%) followed a similar trend and was positively correlated ( $r = +0.59$ ;  $P = 0.05$ ) with percentage diseased plants and high closer to the river (Table 4.4). During the field experiments, no rainfall was recorded in the Ouémé valley. Air temperature and humidity recorded for the duration of the study are presented in Table 4.5. At the susceptible stage of the host plant (first four weeks of growth), the weekly mean air temperatures and humidity varied between 26.1 - 26.3 °C and 69.0 - 75.0 %, respectively in 2001, and between 25.8 - 26.9 °C and 71.1 - 73.6 %, respectively, in 2002.

#### 4.4 Discussion

Results of this study indicated that, of all the fungal species isolated from diseased cowpea plants, only *S. rolfsii* caused damping-off and stem rot of cowpea during the pathogenicity tests in the greenhouse. *S. rolfsii* has been recorded in many countries in Africa, in tropical Asia, in Brazil and in the United States of America (Singh and Rachie, 1985). *S. rolfsii* has been reported to be a destructive soil-borne pathogen of numerous crops, especially in the tropics and subtropics (Mukherjee and Raghu, 1997; Punja, 1985). In the present study, *S. rolfsii* was identified as the causal agent of damping-off and stem rot, as recorded earlier in the Ouémé valley (Adandonon, 2000;

Kossou *et al.*, 2001), in a complex which also included *F. oxysporum*, *F. scirpi*. and *T. harzianum*. *Fusarium oxysporum*, *F. scirpi* and *T. harzianum* isolated in the present study did not individually cause damping-off and stem rot symptoms, indicating that they might not be considered pathogenic on cowpea, as reported earlier on other crops (Mukherjee and Raghu, 1997; Singh *et al.*, 1997; Weideman and Wehner, 1993). The fungal interaction effect experiment showed that *S. rolfsii* isolates paired with *Fusarium* resulted in a significantly lower percentage of diseased plants than when *Sclerotium* was used singly to infect the soil. There may have been a competitive antagonism between the two fungi without tissue death as found in a previous study on PDA (Adandonon, 2000). When *T. harzianum* was paired with *S. rolfsii* isolates, no diseased plants were recorded. *Trichoderma harzianum* may have suppressed the multiplication and growth of *S. rolfsii* as reported by Mukherjee and Raghu (1997) on a ginger rhizome disease in a slice bioassay.

In the naturally infected field experiment, a gradient of *S. rolfsii* initial inoculum was recorded and the highest density was found closest to the river. A similar pattern was obtained for the percentage soil moisture, disease incidence and inoculum density. Punja *et al.* (1985) recorded the same spatial pattern of inoculum of *S. rolfsii* and indicated that the inoculum was clustered or clumped and that a similar trend was observed for diseased plants. In the Ouémé valley, the soil preparation by farmers before planting always comprises the cutting of harvested maize stems and weeds and making holes in which cowpea seeds are directly sown. The stems and weeds are placed on the field during the cowpea development period and are remoistened by dew, thus increasing the amount of organic substrate for mycelial growth. This may lead to increased disease incidence and severity as reported earlier (Beute and Rodriguez-Kabana, 1979; Punja, 1985).

It is common knowledge that environmental conditions may influence the development of disease (Pieczarka and Abawi, 1978). The etiology of a particular plant disease has important implications in the cultivation of the crop and its understanding is crucial in order to be able to control and eliminate the disease before it becomes established in the crop (Smith, 1997). Air temperature and humidity play an important role in the initiation and development of infectious plant diseases (Pieczarka and Abawi, 1978; Wakeham *et al.*, 1997). Diseases caused by *S. rolfsii* are especially rampant in the tropics and subtropics and in areas of the southern and southeastern USA where temperatures are sufficiently high to permit the growth and survival of the fungus (Mukherjee and



Raghu, 1997; Punja, 1985). The temperature for maximum growth and sclerotial formation of *S. rolf sii* ranges from 27-30 °C (Punja, 1985) whilst the optimal temperature for growth and disease initiation is between 30 and 35 °C (Mukherjee and Raghu, 1997). *S. rolf sii* grows and produces sclerotia at or near the soil surface (Punja, 1985) and growth may be influenced more by air than soil temperature. During the present study, air temperature and relative humidity did not vary significantly and might not be the dominant determinators influencing progress of cowpea damping-off and stem rot diseases in the Ouémé valley. However, soil moisture might be one of the important factors influencing disease development in the valley. The fact that there was no rainfall during the two-year field experiment is typical for the production period of cowpea in this study area. Moisture available in the soil after natural flooding was the primary source of moisture that might have influenced disease development. The positive correlation between soil moisture and disease incidence in the present study corresponds with findings on other soilborne diseases (Piecarka and Abawi, 1978). Results from this study give the first documentation on the field factors favouring cowpea damping-off and stem rot development in the Ouémé valley. *Sclerotium rolf sii* initial inoculum and soil moisture were among the main factors influencing disease development during the present study. Results of the present study are important in the context of disease control methods, prediction and development of an infection efficiency model (Lalancette *et al.*, 1988) based on environmental data.

#### 4.5 Acknowledgements

This research was financed by the International Institute of Tropical Agriculture (IITA). We thank to the National Institute of Agricultural Research of Benin (INRAB), the University of Pretoria and the National University of Benin (UNB) for provision of facilities and technical material.

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**Table 4.1**

Pathogenicity of various fungal isolates, applied singularly and in various combinations, in terms of damping-off and stem rot development of cowpea in the greenhouse

Fungal isolates <sup>x</sup>	Inoculum amount (g kg <sup>-1</sup> pasteurised soil)	Diseased seedlings and plants (%) <sup>y</sup>
Sr 1	20	75 c <sup>z</sup>
Sr 2	20	87.5 d
Fo	20	0 a
Fs	20	0 a
Th	20	0 a
Sr 1 + Fo	10 each	60.75 b
Sr 1 + Fs	"	63.25 bc
Sr 1 + Th	"	0 a
Sr 2 + Fo	"	75 c
Sr 2 + Fs	"	71.50 bc
Sr 2 + Th	"	0 a
Sr 1 + Fo + Th	6.7 each	0 a
Sr 1 + Fs + Th	"	0 a
Sr 2 + Fo + Th	"	0 a
Sr 2 + Fs + Th	"	0 a
Sr 1 + Fo + Fs + Th	5 each	0 a
Sr 2 + Fo + Fs + Th	"	0 a
Control 1	0	0 a
Control 2	0	0 a

<sup>x</sup>Interactions were evaluated *in vivo* on damping-off and stem rot of cowpea by inoculating the soil with the single or paired fungi. Sr 1: *S. rolfisii* isolate from Agonguey; Sr 2 *S. rolfisii* isolate from Agonlin; Fo: *F. oxysporum*; Fs: *F. scirpi*; Th: *T. harzianum*. "+" indicated that two, three or four isolates, depending on the case were mixed to inoculate soil; Control 1: pasteurised soil mixed with 20 g uninoculated steeped millet seed; Control 2 = pasteurised soil without steeped millet seed. The diseased plants were recorded at 2-d intervals until 30 d.  
<sup>y</sup>Damping-off and stem rot. <sup>z</sup>Each value is an average of four replicates. Means with the same letters are not different ( $P = 0.05$ ) according to General Linear Model test.

**Table 4.2**

Isolation frequency (%) and pathogenicity test results of fungi associated with cowpea damping-off seedlings and stem rot plants collected from six villages in the Ouémé valley, Benin during 2001 and 2002

Fungal species	Isolation frequency		Number of		Isolates (%) causing	
	(%) <sup>x</sup>		isolates tested <sup>z</sup>		damping-off or stem	
	2001	2002	2001	2002	2001	2002
<i>Fusarium scirpi</i>	2.00 a <sup>y</sup>	5.46 a <sup>y</sup>	19	80	0	0
<i>F. oxysporum</i>	7.23 b	13.59 b	69	100	0	0
<i>Sclerotium rolfsii</i>	100 c	100 c	100	100	100	100
<i>Trichoderma harzianum</i>	4.82 ab	3.38 a	46	50	0	0
Controls	0	0	0	0	0	0

<sup>x</sup>Isolation frequency (%) or percentage of diseased plants from which the fungus was isolated out of a total number of 953 or 1476 diseased plants collected during 2001 and 2002, respectively, from six villages in the Ouémé valley, Benin. <sup>y</sup>Values within a column not followed by the same letters are significantly different ( $P=0.05$ ) according to the General Linear Model test. <sup>z</sup>For pathogenicity tests in the greenhouse, when the number of isolates of a particular fungal species was less than 100, all isolates were tested.

**Table 4.3**

Initial inoculum density of *Sclerotium rolfsii* recorded per field plot planted with cowpea during 2001 and 2002 in Agonguey, in the Ouémé valley, Benin

Distance from the river (m)	Initial inoculum (sclerotia) at planting <sup>x</sup> (Kg <sup>-1</sup> soil)	
	2001	2002
200	24.6 c <sup>y</sup>	34.7 c
400	20.5 bc	25.1 b
600	18.3 b	19.9 b
800	11.3 a	12.4 a
1000	12.6 a	12.2 a

<sup>x</sup>*S. rolfsii* initial inoculum was recorded in the field before planting in 2001 and 2002. <sup>y</sup>Six 1000 g-soil samples were taken from each of the five plots within bands running perpendicular to the river and number of sclerotia determined using the wet-sieving method (16). <sup>y</sup>Means with the same letters are not different ( $P = 0.05$ ) according to the General Linear Model test.

**Table 4.4**

Incidence of damping-off and stem rot of three cowpea cultivars, and soil moisture values per field plot in 2001 and 2002 in Agonguey, in the Ouémé valley, Benin

Distance from the river (m)	Diseased plants <sup>x</sup> (%)					Soil moisture (%)	
	Tchawé kpayo		Tchawé daxo	IT83D-326-2			
	2001	2002	2002	2002	2001	2002	
200	8.51 <sup>y</sup> c	7.28 <sup>y</sup> c	1.32 b	4.37 b	24.81 <sup>y</sup> c	25.43 <sup>y</sup> c	
400	6.54 b	5.86 bc	0.87 b	3.42 b	24.68 bc	23.94 b	
600	4.82 b	4.39 b	1.13 b	1.06 a	23.61 ab	24.03 bc	
800	2.19 a	1.99 a	0.06 a	1.26 a	23.88 abc	22.21 a	
1000	0.8 a	1.04 a	0.01 a	0.53 a	23.53 a	21.11 a	

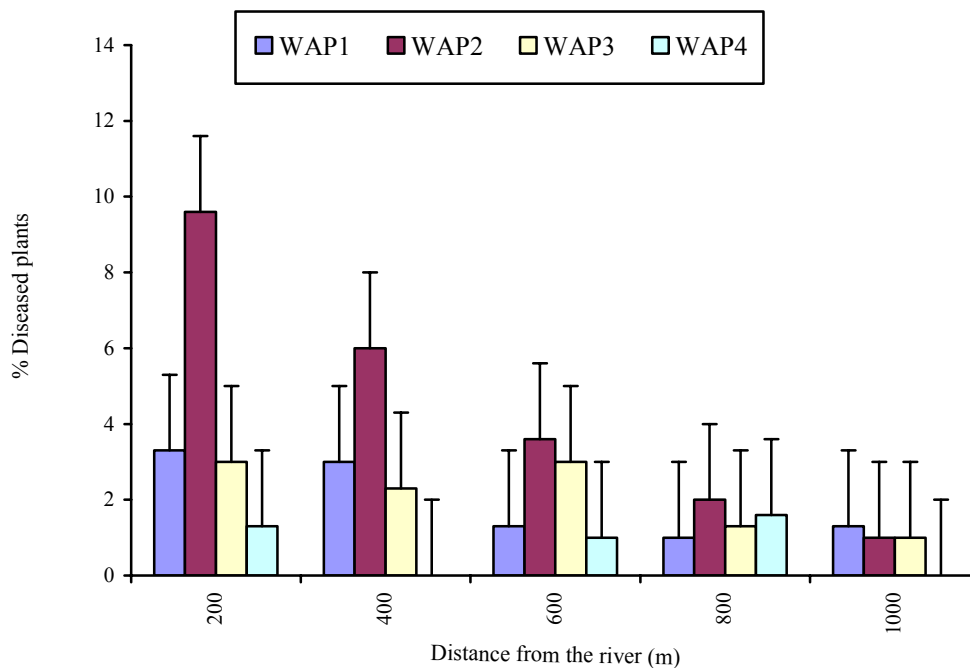
<sup>x</sup>Damping-off seedlings and stem rot plants were recorded during 2001 on Tchawé kpayo (susceptible cultivar) and during 2002 on three cowpea cultivars: Tchawé kpayo, Tchawé daxo (tolerant cultivar) and IT83D-326-2 (moderately tolerant). <sup>y</sup>Means per column, followed by the same letters are not significantly different ( $P = 0.05$ ) according to the General Linear Model test.

**Table 4.5**

Air temperature and relative humidity recorded in cowpea field experiments during 2001 and 2002 in the Ouémé valley, Benin

	Temperature (°C)						Relative humidity (%)					
	Minimum		Maximum		Mean		Minimum		Maximum		Mean	
WAP <sup>x</sup>	2001	2002	2001	2002	2001	2002	2001	2002	2001	2002	2001	2002
1	21.29	21.33 <sup>y</sup>	31.29	30.28 <sup>y</sup>	26.29	25.80 <sup>z</sup>	42.00b	45.23ab <sup>y</sup>	97.00	97.16 <sup>y</sup>	69.50ab	71.19ab <sup>z</sup>
2	21.43	21.12	30.86	31.65	26.14	26.38	53.29bc	49.20ab	97.00	98.00	75.00ab	73.60b
3	21.29	21.64	31.29	32.00	26.29	26.82	42.43b	45.32ab	97.00	98.00	69.00ab	71.66ab
4	21.4	21.43	30.86	31.42	26.14	26.42	52.55bc	45.79ab	97.00	97.00	74.45ab	71.39ab
5	21.14	20.79	31.71	30.74	26.43	25.76	47.86bc	49.24ab	97.71	97.85	72.89ab	73.54b
6	21.43	21.13	30.43	30.49	25.93	25.81	63.14c	58.51b	97.86	96.99	80.50b	77.75c
7	21.71	21.25	30.14	31.81	26.00	26.53	61.43c	49.36ab	97.29	97.39	79.36b	73.37ab
8	21.00	20.82	32.00	32.33	26.50	26.57	46.71bc	52.11b	98.14	98.00	72.43ab	75.05b
9	17.71	19.39	33.14	32.92	25.43	26.15	15.71a	32.18ab	98.57	98.00	57.14a	65.09a
10	18.00	18.40	34.29	33.17	26.14	25.78	15.14a	22.45a	98.71	97.84	56.93a	60.14a

<sup>x</sup>WAP = Weeks after planting. <sup>yz</sup>Each value is an average of 7 and 14 values, respectively. In the same column, means followed by the same letters are not significantly different ( $P = 0.05$ ) according to the General Linear model test.



**Figure 4.1** Incidence of cowpea damping-off and stem rot at 200 m distances from the river at various times in the field in the Ouémé Valley, Benin. Because damping-off and stem rot of cowpea are both caused by *S. rolfsii*, the data for the two disease syndromes were combined and expressed as percentage diseased plants. WAP = weeks after planting. Y error bars are standard deviation values.



## **CHAPTER 5**

# **GENETIC VARIATION AMONG *SCLEROTIUM* ISOLATES FROM BENIN, DETERMINED USING MYCELIAL COMPATIBILITY AND ITS rDNA SEQUENCE DATA**

**GENETIC VARIATION AMONG *SCLEROTIUM* ISOLATES FROM BENIN AND SOUTH AFRICA, DETERMINED USING MYCELIAL COMPATIBILITY AND ITS rDNA SEQUENCE DATA**

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*In press as:*

Adandonon, A., Aveling, T. A. S., van der Merwe, N. A. and Sanders, G. 2004. Genetic variation among *Sclerotium* isolates from Benin and South Africa, determined using mycelial compatibility and ITS rDNA sequence data. *Australasian Plant Pathology*

**Abstract**

Damping-off and stem rot of cowpea caused by *Sclerotium rolf sii*, has previously been reported in Benin, where the pathogen showed variation in growth and sclerotia production among isolates. Pathogenicity, mycelium compatibility group (MCG) tests and DNA sequence analyses were conducted on different isolates of *S. rolf sii* and *S. delphinii* collected from different hosts and geographical areas in Benin and South Africa. All the isolates, when inoculated into soil and planted with cowpea, caused the disease symptoms. Virulence among isolates varied depending on the host of origin. Isolates originating from cowpea produced the highest disease incidence followed by isolates from peanuts. Four MCGs were distinguished among 66 isolates. Isolates from the same hosts tended to group into the same MCG. The incidence of damping-off and stem rot of cowpea, expressed as percentage diseased plants, varied among MCGs. Plants inoculated with MCG2 displayed the highest, while those inoculated with MCG4 the lowest disease incidence. Parsimony analysis of ITS DNA sequence data supported a close affinity of the *Sclerotium* spp. but showed genetic variation among isolates with no grouping based on host origin.

Keywords: *Athelia rolf sii*, PCR, ribosomal RNA, *Sclerotium* rot.

## 5.1 Introduction

*Sclerotium rolfsii* Sacc. (teleomorph: *Athelia rolfsii* (Curzi) Tu & Kimbrough) is a soilborne plant pathogenic fungus that causes disease in over 500 plant species throughout the world (Punja, 1985; Harlton *et al.*, 1995; Cilliers *et al.*, 2000). Recently, *S. rolfsii* was also reported as the causal agent of damping-off and stem rot on cowpea, *Vigna unguiculata* (L.) Walp, in the Ouémé Valley, Benin (Adandonon, 2000; Kossou *et al.*, 2001) where yield losses reached up to 40 % (PEDUNE-BENIN, 1995). In a previous study, variation in growth rate, number and time to first production of sclerotia by *S. rolfsii* isolates on potato dextrose agar (PDA), was observed among isolates collected from different villages in the Ouémé valley (Adandonon, 2000). Also, considerable variability among *S. rolfsii* from Brazil was detected in terms of the number, size and location of sclerotia on the medium surface (Almeida *et al.*, 2001). Two related species, *S. delphinii* and *S. coffeicola*, which are reported to occur on ornamental bulbs and coffee respectively, produce larger sclerotia that are lighter brown to orange in colour (Stevens, 1931; Harlton *et al.*, 1995; Punja and Damiani, 1996). It is not clear whether these differences in host occurrence and morphology of sclerotia are sufficient to warrant a separate species designation for these isolates (Harlton *et al.*, 1995).

Punja and Grogan (1983) found that field isolates of *S. rolfsii* from various hosts and geographical areas, even within the same area, showed variation in growth rate, numbers and size of sclerotia, and mycelium compatibility. More recently, Punja and Sun (2001) detected 68 mycelium compatibility groups (MCGs) among 128 isolates of *S. rolfsii*. Nalim *et al.* (1995) identified 25 MCGs among *S. rolfsii* isolates collected from Texas, and used molecular markers to show that all isolates within an MCG had identical genotypes. Some MCGs also shared the same sequence for the internal transcribed spacer (ITS) region of ribosomal DNA. Molecular markers are increasingly being used to characterise populations of plant pathogens (Michelmore and Hubert, 1987). The polymerase chain reaction (PCR) and DNA sequencing, among others, have been used to study fungal populations (Taylor *et al.*, 1999; Taylor *et al.*, 2000). Since no studies of this nature have been reported on *S. rolfsii* isolates from Benin, the objective of this study was to use pathogenicity, MCGs and DNA sequence analysis to determine the extent of variation amongst *S. rolfsii* and *S. delphinii* isolates collected from diverse hosts and geographical areas in Benin and South Africa.

## 5.2 Materials and methods

### 5.2.1 Fungal isolates and culture maintenance

The isolate designations and other relevant information of the 66 *Sclerotium* spp. isolates included in this study are listed in Table 5.1. Fifty-five were isolates from cowpea in Benin: one each from 33 different sites in Agonguey, 11 sites in Agonlin, six sites in Dannou and five sites in Gangban. Eleven isolates were from South Africa: 10 from peanut and one *S. delphinii* isolate from bambara groundnut (*Vigna subterranea* (L.) Verdc.) in South Africa. Cultures were maintained on potato dextrose agar (PDA) slants and stored at room temperature.

### 5.2.2 Pathogenicity tests

The variation in pathogenicity of the isolates was evaluated using a *Sclerotium*-susceptible cowpea cultivar, Tchawé kpayo (Adandonon, 2000). Soil inoculation was done using the milled seed inoculation technique (Weideman and Wehner, 1993), described in Chapter 3. There were 68 treatments (66 isolates and two controls) arranged in a randomised block design with three replicates. The two controls were pasteurised soil (1 kg) unmixed or mixed with 20 g uninoculated steeped millet seed, respectively. Four cowpea seeds were planted per pot (one replicate) filled with inoculated soil and kept in the greenhouse at temperatures varying between 23 and 30°C. The percentage of damping-off seedlings was recorded after 3 d, followed at 7-d intervals until 30 d after planting.

To fulfil Koch's postulates, dying seedlings were removed at each observation, and at least one plant from each pot was submitted to agar culture assays to verify the presence of *Sclerotium* spp. (Brantner and Windels, 1998). The reisolated fungus was cultured on PDA and colony characteristics recorded and compared to the original isolates. Data representing disease percentages (damping-off and stem rot) were transformed with arcsine transformation (Brantner and Windels, 1998). Analyses were performed using the SAS System (SAS, 1997) and mean separations were done according to the General Linear Model test.

### 5.2.3 Mycelial compatibility groups (MCG)

All isolates were paired against each other on PDA to observe mycelial interactions (Harlton *et al.*, 1995; Cilliers *et al.*, 2000). Three isolates were simultaneously paired on one dish and assigned to different MCGs based on the presence of a barrage zone

between the isolates. Mycelial plugs (6-mm diameter) taken from the edge of 5-7 d-old colonies were placed approximately 25 to 35 mm apart in 100 x 15 mm Petri dishes and incubated at  $29 \pm 1^\circ\text{C}$ . Each pairing was repeated and all isolates were paired with themselves as controls. The pairings were examined macroscopically after 5 d for the presence of barrage zones in the region of mycelial contact (Punja and Grogan, 1983). A pH-sensitive medium ( $50 \text{ mg l}^{-1}$  Bromocresol green) described by Powell (1995) was used to confirm the total number of identified MCGs. Petri dishes with pairing isolates on pH-sensitive medium were incubated for 5 to 7 d at  $25^\circ\text{C}$  in the dark. Incompatible reactions were distinguished by a coloured line in the reaction zone between the colonies, as viewed at a slight angle from the bottom of the plate. Compatible reactions were distinguished by merged colonies with no detectable line. All pairings were performed twice.

#### 5.2.4 Population analysis

Each MCG was assigned a number (MCG1-MCG4). The most common MCG in samples was determined by identifying the number of representatives in each group. The genotypic diversity ( $G_{ST}$ ) of the population was determined as proposed by Stoddart and Taylor (1988). The maximum percentage of genotypic diversity ( $\hat{G}$ ), for the total set of isolates, was determined by dividing the genotypic diversity by the total sample size ( $G_{ST}/N$ ). In the present study,  $\hat{G}$  instead of the Shannon Index ( $SI$ ) was used for comparative purposes.

#### 5.2.5 Preparation of DNA

Representative isolates of each of the MCGs were selected for DNA sequence analysis. Selected isolates (Table 5.1) were grown at  $25^\circ\text{C}$  in 20 ml of nutrient broth enriched with  $16 \text{ g l}^{-1}$  glucose and shaken at 100 rpm. After two weeks, the mycelial mat was harvested, freeze-dried and ground to a fine powder using liquid nitrogen.

DNA preparation was done using the FTA system (Whatman-BioScience, Abington, UK). A small amount of the ground mycelium was applied to the FTA matrix, which was divided into grids for several samples per matrix and left to dry thoroughly. A section with no applied mycelium served as a negative control. A small square (2 mm x 2 mm) was cut from the matrix, placed into a sterile 0.2-ml PCR tube and washed three times with  $100 \mu\text{l}$  of FTA reagent (Whatman-BioScience, Abington, UK) for 5 min. The

matrix was then rinsed twice with 100 µl of sterile distilled water. The FTA discs were vacuum dried, placed on ice and PCR conducted immediately.

#### 5.2.6 PCR and gel electrophoresis

Nineteen representative isolates (Table 5.1) were compared by means of ribosomal DNA internal transcribed spacer sequence analysis, including the 5.8 S gene, using primers ITS1 and ITS4 (White *et al.*, 1990). PCR was done using 50 µl volumes, each containing 10 mM of each dNTP (TaKaRa), 1 x PCR buffer, 20 pmol of each primer (ITS 1 and ITS 4) and 2.5 U of *Taq* polymerase (TaKaRa). Thirty PCR cycles were performed on a Perkin-Elmer 2400 thermocycler using the following conditions: one initial denaturation at 95 °C for 2 min, a denaturation step at 93 °C for 30 sec followed by annealing at 55 °C for 45 sec and extension at 72 °C for 90 sec. PCR was concluded with a final extension at 72 °C for seven minutes. PCR products were visualised using ethidium bromide under ultraviolet light after electrophoresis on a 1.2 % w/v agarose gel at 2 v/cm for 1 h.

#### 5.2.7 Sequencing reaction mix and sequence analysis

DNA sequencing reactions were carried out using an ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction kit (Applied BioSystems) according to the manufacturer's specifications. Analysis of sequences was done using the Phylogenetic Analysis Using Parsimony (PAUP\*) 4.0b10 computer software (Swofford, 1998). A heuristic parsimony analysis with random step-wise addition and tree-bisection-reconnection (TBR) was used to construct a phylogram. The confidence of branches was determined using a bootstrap analysis with 1000 replicates. Previously published sequences of seven *S. delphinii* isolates (GenBank accessions: AB075316, AB075313, AB075311, AB075317, AB075315, AB075314, AB075312) and one *Athelia rolfsii* isolate (AB042626) were included in this study (Okabe and Matsumoto, 2003). Trees were rooted by the outgroup (*S. coffeicola*), represented by sequence data from GenBank (AB075319).

### 5.3 Results

#### 5.3.1 Pathogenicity and incidence

All the *Sclerotium* isolates caused damping-off and stem rot symptoms on cowpea. No lesion development was observed with controls. However, there was variation among

isolates and virulence varied depending on the host of origin. Isolates collected from cowpea showed the highest incidence followed by those from peanuts. The lowest disease incidence was recorded with the *S. delphinii* isolate collected from bambara groundnut.

### 5.3.2 Mycelial compatibility groups (MCGs) and population analysis

During the MCG test, antagonism zones developed between incompatible isolates and mycelium thinned out in the region of interaction. In all selfed pairings, the hyphae fused and no barrage zones developed. Barrage zones always developed between mycelia of isolates that were vegetatively incompatible, whereas compatible isolates had fused mycelia and dense growth associated with abundant sclerotia production in the contact zone. Isolates that were vegetatively compatible were grouped in the same MCG. Four MCGs were identified for the 66 *Sclerotium* isolates: MCG1, MCG2, MCG3 and MCG4 (Table 5.1). The method using a pH indicator confirmed the identification of four MCGs.

The MCG phenotype correlated with host of origin: Cowpea isolates were MCG1 or 2, peanut isolates were MCG3 and the single *S. delphinii* isolate from bambara groundnut was MCG4. Conversely, isolates from one geographical region or even one subregion belonged to several MCGs (Table 5.1). For example, isolates from the subregion Agonguey-Benin were members of both MCG1 and 2. Isolates from South Africa grouped in MCG3 and 4. An MCG could also contain isolates from different areas, for example MCG1 contained isolates from three villages and MCG2 from two. MCG1 was the most frequent (53 %), followed by MCG2 (30 %), MCG3 (15 %) and MCG4 (one isolate).

The genotypic diversity ( $G_{ST}$ ) for the total number of genotypes (MCGs) was 2.307 and the maximum percentage of genotypic diversity ( $\hat{G}$ ) was 3.5 %. Furthermore, the incidence of damping-off and stem rot of cowpea expressed as percentage diseased plants varied among MCGs. MCG2 showed the highest levels of disease incidence (94.2 %), which is significantly ( $P = 0.05$ ) different from that of MCG1 (83.6 %), whereas MCG4 caused the least diseases on cowpea (30.4 %) that was significantly lower than that of MCG3 (41.7 %).

### 5.3.3 ITS-PCR and DNA sequencing

Gel electrophoresis of PCR products yielded a band of approximately 700 bp. Different clades and sub-groups were inferred from the phylogenetic analysis, yielding diverse associations. There was no apparent clustering of isolates according to host or village of origin, although isolates from the same country grouped together. Isolates from cowpea and peanut were in the same clade, but the *S. rolfsii* isolates from cowpea formed a group separate from peanut isolates. This sub-group is supported by a bootstrap value of 62 %. Other subgroups were found and some isolates from the same area (Agonguey) were in a separate subgroup. For example, AGG55, AGG14 and AGG31 were in a clade different from that of other isolates from Agonguey (Fig. 5.1). The one isolate from bambara was in the same clade with *S. delphinii* reference isolates. Moreover, isolates from Benin were in the same major clade with peanut isolates from South Africa and this is supported by a 100 % bootstrap value. The *S. delphinii* (NEL01) isolate from bambara groundnut and *S. delphinii* reference isolates formed a separate subgroup from other *S. rolfsii* isolates. Furthermore, isolates from different MCGs had nucleotide sequence that clustered into the same subgroup. Thus, AGG14 and AGG31 of MCG1 were in the same subgroup as AGG55 of MCG2, indicating that the isolates had similar ITS DNA sequences. This clade is supported by a bootstrap value of 62 %. Some isolates from the same MCG were in different subgroups. For instance, AGG14 and GAN03 from MCG1 were in two different subgroups, and this separation was supported by a bootstrap value of 62 % (Fig. 5.1).

## 5.4 Discussion

In this study, all the *S. rolfsii* and *S. delphinii* isolates caused damping-off and stem rot symptoms on cowpea. However, variation in disease incidence was observed. Pathogenic variation has been well demonstrated in many species of fungi (Punja, 1985; Chulze *et al.*, 2000; Van Heerden and Wingfield, 2001) including *Sclerotium* spp. (Punja, 1985).

Results from this study showed that the isolates from Benin were represented by two MCGs, as were those from South Africa. Only four MCGs were identified within the 66 isolates collected from different hosts and geographic areas. The method described by Powell (1995) resulted in the same MCG groupings that were observed on PDA medium. Harlton *et al.* (1995) screened a worldwide collection of *S. rolfsii* isolates and identified 49 MCGs from 119 isolates. Similarly, Punja and Grogan (1983) showed that



*S. rolfsii* could be placed in MCGs based on mycelial interactions and they identified 25 groups from 72 isolates. Moreover, 13 MCGs were identified among 23 *S. rolfsii* isolates collected from different hosts and regions of Brazil (Almeida *et al.*, 2001). In the current study, however, the four MCGs detected among the 66 isolates indicates a low level of phenotypic differentiation.

The fact that isolates originating from the same area within the Ouémé valley were placed in different MCGs suggests occurrence of genetic differences within the subpopulations due to migration. Basidiospores may be involved in spread since colonies arising from basidiospores would be variable enough to be in a new MCG (Punja and Grogan, 1983; Harlton *et al.*, 1995). Carlile (1986) suggested that a single MCG could predominate in an area if a fungal isolate colonised a new area or host and then spread vegetatively. Also, the low number of MCGs could suggest that this species was introduced relatively recently (Harlton *et al.*, 1995). If genetic divergence occurs over time among individuals, through recombination and migration for instance, new MCGs could result. In contrast, the recovery of a particular MCG of *S. rolfsii* in widely different geographic areas may have resulted from spread because of agricultural practices (Harlton *et al.*, 1995). Therefore, the two MCGs found in Agonguey in the present study could be attributed to migration. The very low number of MCGs found in this study could suggest that either the introduction of *S. rolfsii* is very recent in Benin, or the type of reproduction is asexual with a low level of migration as often happens with this fungal species (Harlton *et al.*, 1995).

Based on the MCGs, the genotypic diversity ( $G_{ST}$ ) for the studied isolates was estimated at 2.3. Genotypic diversity is a reliable measure that can be used to compare data from different pathosystems with different sample sizes (Milgroom *et al.*, 1992; McDonald and McDermott, 1993). Therefore, the *Sclerotium rolfsii* population of the present study was genetically uniform, compared to previous reports on the pathogen (Punja and Grogan, 1983; Harlton *et al.*, 1995; Cilliers *et al.*, 2000; 2002).

DNA sequence analyses showed diverse clades and sub-groups that did not correspond to host or origin of occurrence of the isolates. These results are similar to those of Cilliers *et al.* (2000) and Harlton *et al.* (1995). Cilliers *et al.* (2000) compared MCGs and ITS regions among isolates of *S. rolfsii* and reported that there was no apparent clustering according to host or geographic origin for the MCGs. Similarly, Harlton *et al.* (1995) found that unique individuals were not necessarily correlated to the host nor restricted in geographical range, and that clonally derived isolates within an MCG

appeared to share ITS restriction sites. The latter also indicated that members within one MCG that were subjected to different evolutionary constraints, could possess the same vegetative compatibility alleles but different ITS sequences. In our study, isolates from different MCGs had nucleotide sequences that clustered into the same clade. The highly similar genetic backgrounds among isolates from different MCGs could differ at only one or few genetic loci that determine cultivar specificity, or specific virulence or pathogenicity (Dobinson *et al.*, 1998).

ITS-DNA sequence analyses showed that all *S. rolfsii* isolates grouped together with *Athelia rolfsii* and some *S. delphinii* reference isolates. In an early ITS study, a close relationship was detected between *S. delphinii* and *S. rolfsii* (Harlton *et al.*, 1995). These two species are currently differentiated based on sclerotial morphology and host range with *S. delphinii* producing the largest sclerotia (Stevens, 1931). Harlton *et al.* (1995) reported that *S. rolfsii* and *S. delphinii* grouped together but separately from *S. coffeicola*. Moreover, Okabe *et al.* (2000; 2003) constructed a phylogenetic tree based on ITS-RFLP analysis and found a close relationship between *S. rolfsii* and *S. delphinii*. Based on their similarity, *S. rolfsii* was designated as *S. rolfsii* var *rolfsii* and *S. delphinii* as *S. rolfsii* var *delphinii* (Boerema and Hamers, 1988; Harlton, 1995). Therefore, results in the current study, could indicate that there is a close affinity of the *Sclerotium* spp. but showed diversity among isolates with no grouping based on host or geographic origin.

In this study, we were able to gain significant insight into the variability among isolates of *S. rolfsii* and *S. delphinii* from Benin and South Africa. More isolates would be required to determine the full extent of genetic diversity among the *Sclerotium* spp. and particularly within *S. delphinii* isolates. However, the possibility exists that the *S. rolfsii* isolates from Benin and South Africa added to the phylogenetic tree in this study, may represent a new pathovar and this will be investigated in future.

## 5.5 Acknowledgments

This work was financed by the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. Thanks are due to Dr AJ Cilliers and Dr J Ade.

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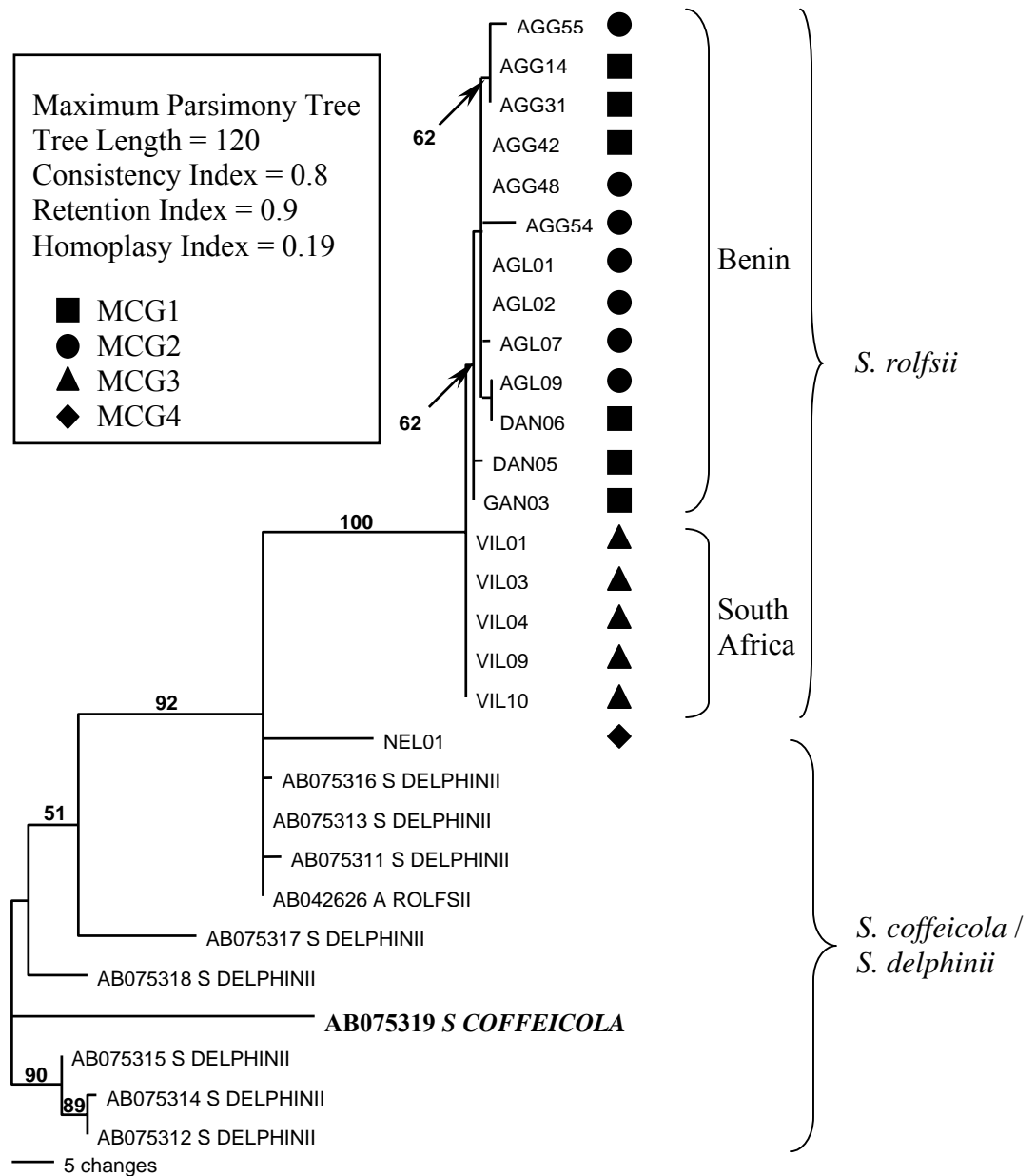
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**Table 5.1**

 Designations, areas, mycelium compatibility group (MCG) and incidence on cowpea in a greenhouse of *S. rolfsii* and *S. delphinii* isolates from Benin and South Africa

MCG	Designation number of isolates <sup>1</sup>	Areas of isolation	Incidence ± SE <sup>2</sup> (%)	MCG	Designation number of isolates <sup>1</sup>	Areas of isolation	Incidence ± SE <sup>2</sup> (%)
1	Agg-10	Agonguey-OB	83.33 ± 8.33	2	<b>Agl-01</b>	Agonlin-OB	91.67 ± 8.33
1	Agg-11	"	83.33 ± 0.00	2	<b>Agl-02</b>	"	100.0 ± 0.00
1	Agg-12	"	83.33 ± 7.71	2	Agl-03	"	91.67 ± 8.33
1	Agg-13	"	75.00 ± 0.00	2	Agl-04	"	100.0 ± 0.00
1	<b>Agg-14</b>	"	83.33 ± 8.83	2	Agl-05	"	91.67 ± 8.33
1	Agg-15	"	75.00 ± 0.00	2	Agl-06	"	100.0 ± 0.00
1	Agg-20	"	75.00 ± 0.00	2	<b>Agl-07</b>	"	100.0 ± 0.00
1	Agg-21	"	83.33 ± 8.83	2	Agl-08	"	100.0 ± 0.00
1	Agg-22	"	91.67 ± 8.33	2	<b>Agl-09</b>	"	91.67 ± 8.33
1	Agg-23	"	83.33 ± 8.83	2	Agl-10	"	91.67 ± 8.33
1	Agg-24	"	83.33 ± 8.83	2	Agl-11	"	100.0 ± 0.00
1	Agg-30	"	75.00 ± 16.7	1	Dan-01	Dannou-OB	83.33 ± 8.33
1	<b>Agg-31</b>	"	91.67 ± 8.33	1	Dan-02	"	83.33 ± 8.33
1	Agg-32	"	91.67 ± 8.33	1	Dan-03	"	83.33 ± 8.33
1	Agg-33	"	83.33 ± 8.33	1	Dan-04	"	91.67 ± 8.33
1	Agg-34	"	91.67 ± 8.33	1	<b>Dan-05</b>	"	91.67 ± 8.33
1	Agg-35	"	75.00 ± 0.00	1	<b>Dan-06</b>	"	83.33 ± 8.33
1	Agg-36	"	83.33 ± 8.33	1	<b>Gan-03</b>	Gangban-OB	75.0 ± 16.70
1	Agg-37	"	83.33 ± 8.33	1	Gan-05	"	75.0 ± 16.70
1	Agg-41	"	83.33 ± 8.33	1	Gan-06	"	83.33 ± 8.33
1	<b>Agg-42</b>	"	91.67 ± 8.33	1	Gan-09	"	91.67 ± 8.33
1	Agg-43	"	75.00 ± 16.70	1	Gan-12	"	75.00 ± 0.00
1	Agg-44	"	91.67 ± 8.33	3	<b>Vil-01</b>	Viljoens-SA	50.00 ± 6.40
1	Agg-45	"	91.67 ± 8.33	3	Vil-02	"	41.67 ± 8.33
2	Agg-46	"	91.67 ± 8.33	3	<b>Vil-03</b>	"	33.33 ± 8.33
2	Agg-47	"	100.0 ± 0.00	3	<b>Vil-04</b>	"	41.67 ± 8.33
2	<b>Agg-48</b>	"	100.0 ± 0.00	3	Vil-05	"	33.33 ± 8.33
2	Agg-49	"	91.67 ± 8.33	3	Vil-06	"	50.00 ± 6.4
2	Agg-52	"	91.67 ± 8.33	3	Vil-07	"	33.33 ± 8.33
2	Agg-53	"	91.67 ± 8.33	3	Vil-08	"	33.33 ± 8.33
2	<b>Agg-54</b>	"	83.33 ± 8.33	3	<b>Vil-09</b>	"	58.33 ± 8.33
2	<b>Agg-55</b>	"	91.67 ± 8.33	3	<b>Vil-10</b>	"	41.67 ± 8.33
2	Agg-57	"	83.33 ± 7.71	4	<b>Nel-01</b>	Nelspruit-SA	30.42 ± 5.71
	Control 1		00.00 ± 0.00		Control 2		00.00 ± 0.00

<sup>1</sup>Sixty six isolates of *S. rolfsii* and one isolate of *S. delphinii* collected from different hosts and geographical areas. All isolates were collected during 2000 from cowpea except those from Viljoenskroon and Nelspruit collected in 2002 from peanuts and bambara groundnut, respectively. Isolate numbers in bold were those selected for PCR amplification and DNA sequencing. Agg, Agl, Dan and Gan represent *S. rolfsii* isolates collected from cowpea from Agonguey, Agonlin, Dannou and Gangban, respectively, in the Ouémé valley, Benin (OB); Vil represents *S. rolfsii* isolates from peanut in Viljoenskroon (Viljoens) in South Africa (SA) and Nel represents *S. delphinii* for the isolate from bambara groundnut in Nelspruit, South Africa. <sup>2</sup>All isolates were pathogenic on cowpea (cultivar Tchawé kpayo), regardless of host origins, in the greenhouse experiment. Incidence is expressed as percentage diseased plants; SE = standard error at 5 % according to the General Linear Model test.



**Figure 5.1** Phylogenetic tree of ITS sequences from *Sclerotium rolfsii* and *S. delphinii*, using the PAUP\* program. The numbers at the nodes are bootstrap percentages. Previously published sequences of seven *S. delphinii* isolates (AB075316, AB075313, AB075311, AB075317, AB075315, AB075314, AB075312) and one *Athelia rolfsii* isolate (AB042626) were included in this study. *S. coffeicola* (AB075319) was used as an outgroup (Okabe and Matsumoto, 2003). Agg, Agl, Dan and Gan represent isolates from cowpea in Agonguey, Agonlin, Dannou and Gangban, respectively, in the Ouémé valley, Benin; Vil represents isolates from peanut in Viljoenskroon in South Africa and Nel for the isolate from bambara groundnut in Nelspruit, South Africa.

## **CHAPTER 6**

# **A NEW LABORATORY TECHNIQUE FOR RAPID SCREENING OF COWPEA CULTIVARS FOR RESISTANCE TO DAMPING-OFF AND STEM ROT CAUSED BY *SCLEROTIUM ROLFSII***



**A NEW LABORATORY TECHNIQUE FOR RAPID SCREENING OF  
COWPEA CULTIVARS FOR RESISTANCE TO DAMPING-OFF AND STEM  
ROT CAUSED BY *SCLEROTIUM ROLFSII***

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**Abstract**

Damping-off and stem rot are major constraints to cowpea cultivation in many countries. Resistant cultivars may be the best control measure to limit the development of the disease in the field. The objective of the study was to determine a rapid laboratory technique to screen cowpea cultivars for resistance to *Sclerotium rolfsii* infection. Two different screening methods were developed and tested in the laboratory: a PDA-based method where cowpea seeds were planted on PDA in tubes, and a paper-based method where seeds were planted on paper pieces in tubes. Correlation and principal component analysis were computed to assess interactions among the different biotic variables of the system. In most cases, diseased seedlings induced by the paper-based method were significantly lower than those by the PDA-based method. Laboratory disease incidence using the paper-based method was positively correlated with the greenhouse ( $r = + 0.68^*$ ) and field incidence ( $r = + 0.62^*$ ). Twelve days after planting (or 8 d after inoculation) on PDA, only two cultivars showed a growth rate higher than 1 mm/d. In contrast, in the paper-based method, the growth rate at 8 d after inoculation correlated ( $r = - 0.91^*$ ) better with the field incidence. In this paper-based method, cultivars with a growth rate higher than 2 mm/d at 8 d after inoculation included all known resistant cultivars with the lowest disease incidence in the two-year experiment in the field, indicating that the paper-based method could be used as a laboratory rapid screening technique.

Keywords: Cowpea, laboratory, greenhouse and field screening.

## 6.1 Introduction

Cowpea, *Vigna unguiculata* (L.) Walp. is an important grain legume in West Africa (Singh *et al.*, 1997). It is eaten in different forms (Quass, 1995) and provides an inexpensive source of protein for the urban and rural poor population (Alghali, 1991). Unfortunately, cowpea yield is low due to susceptibility to many insects and diseases (Emechebe and Florini, 1997) including damping-off and stem rot caused by different fungi such as *Pythium* spp. and *Sclerotium rolfsii* Sacc. (Emechebe and Florini, 1997; Aveling and Adandonon, 2000).

Plant resistance is often referred to as the best feasible method of controlling plant diseases (Singh and Rachie, 1985). Breeding cowpea varieties with resistance to fungal pathogens has been a major goal of many improvement programmes since the early part of the century (Singh and Rachie, 1985). Resistant varieties are often identified through screening methods (Singh and Rachie, 1985). The International Institute of Tropical Agriculture (IITA) developed reliable and fast screening techniques for studies and evaluation of *Striga* and *Alectra* segregating materials (Singh *et al.*, 1997). Fery and Dukes (1986) reported from field screening experiments that there was genetic variability in cowpea and some cultivars were more resistant to *Sclerotium* diseases. In cultivar screening trials in the field in the Ouémé valley, Benin, variation in resistance was detected among cowpea cultivars and K VX-396-18 was more tolerant than the other tested cultivars (Adandonon, 2000). All these screening methods included field and/or greenhouse screenings, using a pot culture technique to test the field screening results and are therefore time consuming.

Since cowpea is mostly utilised as food by small-scale farmers in many countries, a quicker and easier method of screening cultivars against pathogen infections, before cultivars are dispersed, is necessary. A fast, simple and accurate screening method that can be used to identify sources of tolerant or partially resistant cowpea germplasm would help in the development of optimal control of plant pathogens (Pazdernik *et al.*, 1997). The objectives of the present study were to develop and compare two different laboratory screening methods and to determine, using different biotic variables, which of the two could be considered as a rapid screening method of cowpea cultivars for resistance to *S. rolfsii* infection.

## 6.2 Materials and Methods

### 6.2.1 Fungal culture

A highly virulent isolate of *Sclerotium rolfsii* (IITA 408, International Institute of Tropical Agriculture, Cotonou, Benin) isolated from infected cowpea plants in the field in the Ouémé valley, Benin was used. The isolate was plated on potato dextrose agar (PDA) and incubated at  $25\pm 1^\circ\text{C}$ . Cultures were maintained on PDA at  $4^\circ\text{C}$ .

### 6.2.2 Disease incidence and seedling growth rate in relation to the screening methods in the laboratory

#### *Screening methods and seed planting*

Two screening methods, namely PDA- and paper-based methods, were tested. In the PDA-based method, PDA medium was poured to a height of 4 cm in each test tube (15 cm x 2.5 cm) and left to cool before the seeds were planted.

In the paper-based method, paper towel was cut into pieces (ca diameter. 5 mm) and placed in test tubes to a height of 4 cm. Test tubes with pieces of paper were autoclaved before seed planting. The paper pieces in the test tube were watered using a rubber tube (internal diam. 3 mm). One of the tube end was embedded in the layer of the paper pieces and the other end received water that went through the pipe to wet the paper pieces from the bottom of the test tube.

Twenty cowpea cultivars were included in the study. Cultivar characteristics are shown in Table 6.1. Seeds of the various cowpea cultivars were surface disinfected in 1 % sodium hypochlorite solution for 5 min, rinsed in SDW and planted on PDA or on the 4 cm-layer of the paper pieces in the test tubes.

#### *Inoculum and inoculation*

In the PDA-based method, a 5-mm diam. disc cut from the edge of an actively growing 5-d-old *S. rolfsii* culture was placed adjacent to a 4-d old seedling growing on PDA in a test tube. Seedlings on PDA without mycelium discs served as controls. In the paper-based method, pieces of twig (15 mm length) were sterilised and deposited on PDA in petri dishes. One 5-mm diameter disc of *S. rolfsii* culture was placed on PDA with the twig pieces. The culture with twig was incubated at  $29\pm 1^\circ\text{C}$  and after 5 d the twig was used as source of inoculum. Seedlings growing on the towel paper pieces layer in the test tubes were inoculated using *Sclerotium*-inoculated twigs placed adjacent to the seedlings. Seedlings growing on the paper pieces layer in the tube without inoculated

twigs were considered as controls. Tubes were sealed and incubated at  $29 \pm 1$  °C for 12 d.

#### *Experimental design and data collection*

The experimental design was a randomized block design with four replications. One replicate consisted of three test tubes of each cultivar per treatment, and one cowpea seedling was grown in each test tube. The number of diseased seedlings was recorded 5 d after seedling inoculation. Seedlings were observed at 4-d intervals until 12 d after planting (or 8 d after inoculation) and seedlings growth (height) was linearly measured at each observation. The growth rate was calculated as follows:

$$GR_j = (G_j - G_{j-4})/4$$

Where  $GR_{jth}$  and  $G_j$  are the seedling growth rate and growth, respectively, at  $j$  days;  $j$  values are 4, 8 and 12;  $G_0 = 0$  (at planting).

### 6.2.3 Greenhouse screening

#### *Soil inoculation, experimental design and planting*

Disinfected soil was inoculated using the millet seed inoculum technique (Weideman and Wehner, 1993), described in Chapter 3. The experimental design was a randomised block design with 20 cultivars, two controls and four replicates. Control 1 = pasteurised soil mixed with 17 g uninoculated steeped millet seed and Control 2 = pasteurised soil without steeped millet seed. Twenty cowpea cultivars (Table 6.1) were used and seeds were surface disinfected as described earlier and then planted in pots (14 cm diam. x 18 cm height) at four seeds per pot filled with inoculated soil, as described earlier (Chapter 3). Pots were maintained in the greenhouse at 24 to 30 °C and watered regularly.

#### *Data collection and Koch's postulates*

The number of seedlings showing symptoms of damping-off and stem rot were recorded 4, 8, 12, 20 and 30 d after planting. To fulfil Koch's postulates, samples of dying seedlings or plants were removed at each observation and at least one plant from each pot was assayed to verify the presence of the causal agent. The re-isolated fungus was cultured on PDA and colony characteristics were recorded and compared to the original isolates.

#### 6.2.4 Field screening

The 20 cowpea cultivars were evaluated for their level of resistance in a field naturally infected with the pathogen during 2001. The experimental design was a randomised block design with 20 cultivars and four replicates and was repeated in 2002. The susceptible (Tchawé kpayo) and tolerant (Tchawé daho) cultivars in the studied zone were included in the trial and considered as controls. A plot of 15 x 10 m<sup>2</sup> size was assigned to each cultivar. Plots were at 1-m intervals to each other whereas the blocks were demarcated at 200-m intervals to each other. All other cultural practices used (plant density, weeding, etc) were those of the local farmers. The number of damping-off seedlings and stem rot plants were recorded 7 d after planting and at 7-d intervals until 28 d after planting.

#### 6.2.5 Relationship between screening method data from the laboratory, greenhouse and field

The statistical analysis was done as described in Chapter 3. Principal component analysis (PCA) was performed with incidence variables from greenhouse/field and growth variables from laboratory screening methods. The first two principal components (PC) eigenvalues accounted for more than 75 % of the variation in the system, so interpretations were limited to these two principal component axes only. The PC2 was plotted against PC1 to get the relationship among cultivars. Correlation analysis was computed to assess interactions between and among the different biotic variables of the system. For the correlation analysis, significant results at  $P = 0.05$  are indicated in the test with  $r^*$ .

### 6.3 Results

#### 6.3.1 Disease incidence and seedling growth rate in relation to the screening methods in the laboratory

Most of the cultivars, regardless of the screening method, were infected by the pathogen and showed disease incidence that was higher than 30 % (Fig. 6.1). In most cases, percentage diseased seedlings yielded by PDA-based method were significantly higher ( $P = 0.05$ ) than those of the paper-based method. Four (Cameroon, Sèwè, Ife brown and TVU-72-74) and only two (Kumassi and TVX-15084) cultivars showed diseased seedlings lower than 60 % with the paper-based and PDA-based method, respectively.

Although all cultivars were attacked by the pathogen in both screening methods, the recorded seedling growth rate, however, was uneven and variation appeared between the two methods. For instance, 8 d after seedling inoculation on PDA, only six cultivars still grew but only two (Kpodji and Kumassi) showed a growth rate that was higher than 1 mm/d (Fig. 6.2). In contrast, 8 d after seedling inoculation using the paper-based method, eight cultivars (Cameroon, Kpodji, Gboto, Sèwé, Kumassi, Tchawé daho, Ife brown and K VX 396-18) showed a growth rate that was significantly higher ( $P < 0.05$ ) than 2 mm/d (Fig. 6.2).

### 6.3.2 Disease incidence in the greenhouse and field experiments

In the greenhouse, Sèwé, Kpodji, Kumassi and Cameroon, showed the significantly ( $P = 0.05$ ) lowest disease incidence followed by Gboto and K VX-396-18. The highest disease incidence was recorded in Tchawé kpayo (Table 6.2).

In the field experiment, the lowest disease incidence was recorded with Cameroon, Kpodji, Gboto and Sèwé followed by Kumassi, Tchawé daho, Ife brown and K VX-396-18 (Table 6.2). Tchawé kpayo, IT89KD-374-57, IT88M-867-11, TVU-72-74 and K VX-61-1 showed the highest disease incidence in the field. A similar trend was recorded during the second year experiment in the field.

### 6.3.3 The relationship among screening method data from the laboratory, greenhouse and field

Results (Table 6.3) indicated that greenhouse and field data were positively correlated ( $r = 0.89^*$ ). Laboratory incidence using the paper-based screening method positively correlated with greenhouse ( $r = 0.68^*$ ) and field incidence ( $r = 0.62^*$ ). Laboratory incidence using PDA-based screening method did not correlate with field incidence. Furthermore, growth rate at 8 and 12 d after planting using the paper-based method in the laboratory correlated with the field disease incidence. The growth rate at 12 d using the paper-based method correlated better ( $r = -0.91^*$ ) with field incidence. The tested cowpea cultivars grouping based on these two variables (growth rate at 12 d using paper-based method and field disease incidence) was similar (Fig. 6.2 and Table 6.2).

Results were evaluated by transforming original interrelated variables into new uncorrelated variables as principal components derived from the correlated matrix (Table 6.3). The first two principal components gave 75 % of the variation in the system. Growth rate at 12 d using the paper-based method and incidence in the

greenhouse and field were better correlated with the first principal component. Based on the first two principal component scores, cultivars at the positive X axis side corresponded to the eight cultivars that showed growth rate significantly higher ( $P < 0.05$ ) than 2 mm/d using the paper-based screening method (Fig. 6.2) with the lowest disease incidence in the field.

#### 6.4 Discussion

In the present study, two screening methods namely PDA- and paper-based methods were tested in the laboratory and results compared to data from greenhouse and field. The growth rate measured 8 d after seedling inoculation in the PDA-based method yielded only two cultivars that still grew but at a rate lower than 2 mm/d. In contrast, in the paper-based method, eight cultivars showed a growth rate that was significantly higher than 2 mm/d at 8 d after inoculation. These eight cultivars were those with the lowest disease incidence during the experiment in the field. Among these eight cultivars, K VX-396-18, Tchawé daho and Gboto were identified in previous work as tolerant (PEDUNE-BENIN, 1995; Adandonon, 2000). Tchawé kpayo showing the highest disease incidence confirmed an earlier finding reporting the susceptibility of this cultivar to *S. rolf sii* damping-off and stem (PEDUNE-BENIN, 1995; Adandonon, 2000). Furthermore, the eight cultivars corresponded with those grouped using the principal component analysis. These results suggest that the paper-based method described here could be used as a rapid method to screen resistant cowpea cultivars against *Sclerotium* damping-off and stem. Although the PDA-based method gave some grouping, this method has the disadvantage of favouring the growth of many fungal species that develop on PDA. Tubes with seedlings growing together with fungi before inoculation were discarded and as consequence, it was fastidious to have the required number of tubes with seedlings free of growing fungi before inoculation. In contrast, in the paper-based method, the grouping of eight cultivars in the laboratory based on the growth rate at 8 d after inoculation was consistent with the principal component analysis, and correlated well with incidence in the greenhouse and field. Furthermore, very few fungi developed together with seedlings before inoculation in the laboratory, and therefore it was easy to remove them and conduct the paper-based method experiment with the required number of tubes.

In cowpea, few methods have been developed for screening against soilborne diseases. Examples were the screening in the greenhouse (Nwapa and Ikotun, 1988) and field



(Fery and Dukes, 1986) for resistance of cowpea cultivars against *S. rolf sii*. In the greenhouse method, plants must be wounded before inoculation with sclerotia. In other crops and pathogens, other screening methods have been developed (Jimenez and Lockwood, 1980; McBlain *et al.*, 1991; Olah and Schmitthenner, 1985), including the mycelium-inoculum-layer (Walker and Schmitthenner, 1984) and hypocotyls-injection method (Hass and Buzzell, 1976) for screening of soybean (*Glycine max* (L.) Merr. cultivars against *Phytophthora soja* H. J. Kaufman and J. W. Gerdemann. These methods, because they damage the plant stem, are said not to be appropriate for thin stem plants such as soybean (Pazdernik *et al.*, 1997) and cowpea. A recent screening method was developed, called agar plug-inoculation method, which could be used on thin-stemmed seedlings (Pazdernik *et al.*, 1997). This method has also been used to screen soybean cultivars for resistance against *P. soja*, and consisted in placing single plug, mycelial-side down on a cotyledon immediately adjacent to the stem of each seedling. The method was said to be better than the methods formerly used (Pazdernik *et al.*, 1997). However, there is still problem since the method requires a plastic dome for preventing seedlings from heat buildup inside the plastic. Moreover, it takes 17 d to come up to the result evaluation. The method developed in the current study required cheap available materials, and is quick, as it could be completed within 12 days in the laboratory. This is the first report showing a rapid laboratory method to screen cowpea cultivars against damping-off and stem rot.

The results in the present study suggest that the paper-based screening method could be used as a rapid laboratory screening method in determining, within 12 d, cowpea cultivars resistant or tolerant to *S. rolf sii* infections. This is very important since it is cheaper, requires no special skills, avoids the classical strategies that follow long term screening trials both in the greenhouse and field as used in previous trials on cowpea resistance to *S. rolf sii* (Fery and Dukes, 1986; Nwakpa and Ikotun, 1988).

## 6.5 Acknowledgements

This work was financed by the International Institute of Tropical Agriculture (IITA). We are grateful to Mr Sam Korie for data statistical analysis.

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**Table 6.1**

Characteristics of 20 cowpea cultivars used during screening for resistance against damping-off and stem rot

Cultivars	Colour	Origin	Status/References <sup>x</sup>
Cameroon	Red-brown	Cameroon local	NK
Kpodji	Black	Benin local	NK
Gboto	Red	Benin local	T (PEDUNE-BENIN, 1995)
Sèwé	Black	Benin local	NK
Kumassi	Red	Ghana local	NK
IT89D-326-2	White	IITA	M T (Adandonon, 2000)
KVX-396-18	White	Burkina Faso	T (Adandonon, 2000)
IT85F-867	Red	IITA	NK
Tchawé daho	White	Benin local	T (PEDUNE-BENIN, 1995)
Ifè brown	Cream	Nigeria	NK
TVU-15084	Reddish	Nigeria	NK
TVU72-74	White	Nigeria	NK
IT90K-109	Cream	IITA	NK
Sanzi	Brown	Ghana	NK
KVX-61-1	White with cream spot	Burkina Faso	NK
Délékinwa	Cream	Igana, Benin	NK
Tchawé kpayo	White	Benin local	S (PEDUNE-BENIN, 1995)
IT89KD-374-57	White	IITA	NK
IT86D-719	White	IITA	NK
IT88M-867-11	White-reddish	IITA	NK

<sup>x</sup>NK = Not known; T = Tolerant; MT = Moderately tolerant; S = Susceptible.

**Table 6.2**Disease incidence in 20 *Sclerotium*-inoculated cowpea cultivars in the greenhouse and field

Cultivars <sup>x</sup>	Disease incidence (%)	
	Greenhouse <sup>y</sup>	Field <sup>y,z</sup>
Control1	0.00 a	-
Control2	0.00 a	-
Sèwé	12.50 ab	02.9 <sup>y</sup> a
Kpodji	18.75 bc	03.2 a
Kumassi	25.00 bcd	03.2 a
Cameroon	25.00 bcd	04.7 ab
Gboto	31.25 cde	05.5 b
KVX-396-18	31.25 cde	05.5 b
Ife brown	37.50 de	05.9 b
Tchawé daho	43.75 e	06.2 b
Sanzi	62.50 f	08.4 c
IT89D-326-2	62.50 f	08.4 c
Délékinwa	68.75 fg	09.8 cd
IT85F-867	68.75 fg	10.2 cd
IT90K-109	75.00 fgh	10.9 de
IT86D-719	75.00 fgh	11.1 de
TVU-15084	75.00 fgh	11.2 de
KVX-61-1	75.00 fgh	12.4 ef
IT89KD-374-57	81.25 gh	12.4 ef
TVU72-74	81.25 gh	14.3 fg
IT88M-867-11	81.25 gh	14.3 fg
Tchawé kpayo	87.50 h	14.7 g

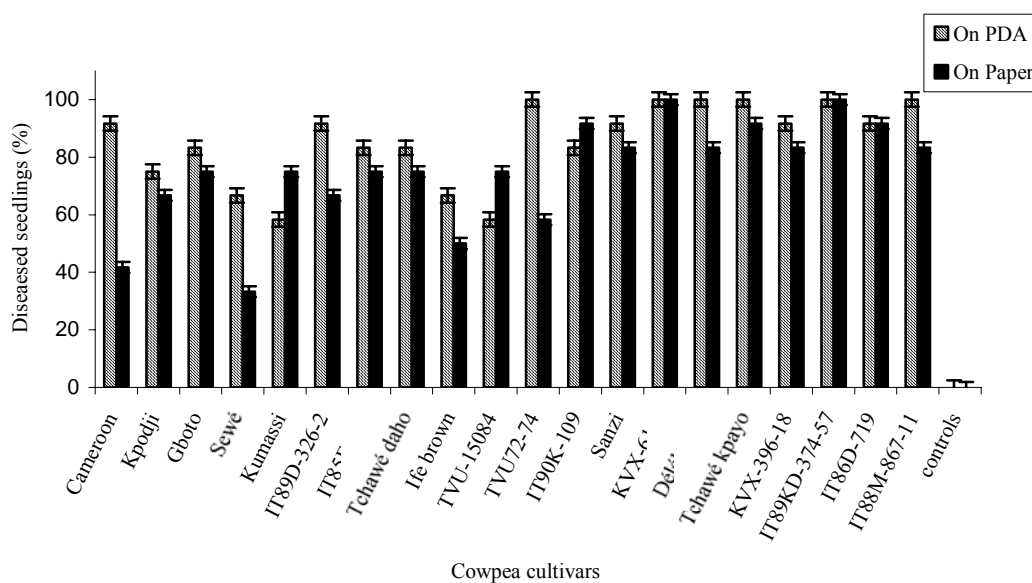
<sup>x</sup>Control 1 = pasteurised soil mixed with 17 g uninoculated steeped millet seed; Control 2 = pasteurised soil without steeped millet seed. In the field, the susceptible (Tchawé kpayo) and tolerant (Tchawé daho) cultivars in the studied zone were included in the trial and considered as controls <sup>y</sup>Disease incidence expressed as percentage diseased plants in the greenhouse and field. In the same column, means follow by the same letter are not significantly different ( $P < 0.05$ ) according to the General Linear Model. <sup>z</sup>Values are the means of two seasons (2001 and 2002).

**Table 6.3**

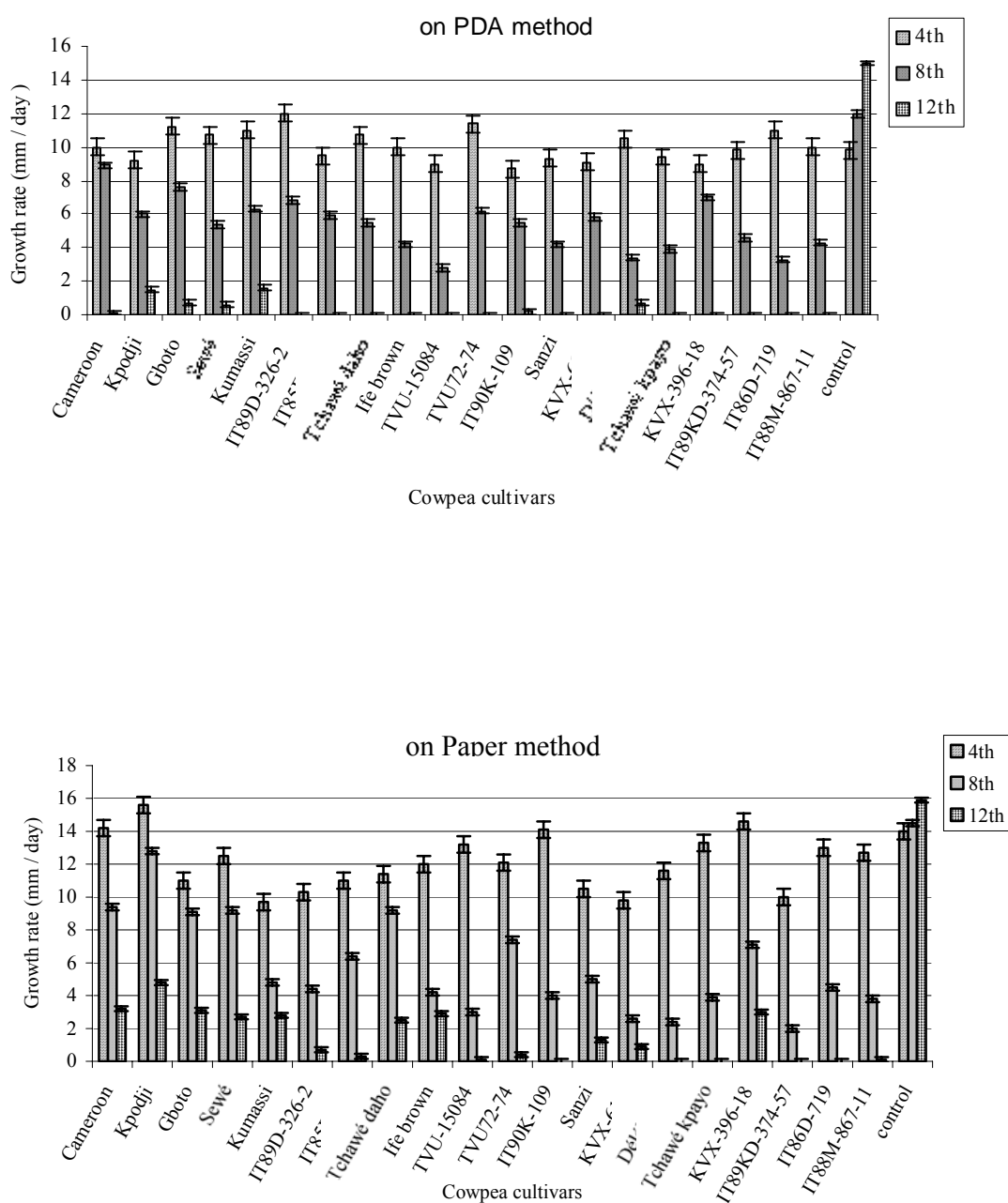
Correlation matrix from parameters measured in the laboratory, based on a PDA and paper towel screening methods and in the greenhouse and field

Variables	Incidence on PDA	Incidence on Paper	Incidence in Greenhouse	Incidence in the field
Incidence on PDA				
Incidence on Paper	0.4124			
Incidence in Greenhouse	0.5337*	0.6873*		
Incidence in the field	0.4923	0.6215*	0.8874*	
Growth rate at 4 d on PDA <sup>x</sup>	0.0911	-.2900	-.1028	-.1585
Growth rate at 8 d on PDA	0.0681	-.3664	-.5155	-.6080*
Growth rate at 12 d on PDA	-.4769	-.1550	-.6183*	-.5548*
Growth rate at 4 d on paper	-.0587	-.2126	-.2484	-.3107
Growth rate at 8 d on paper	-.2003	-.5541*	-.7347*	-.8040*
Growth rate at 12 d on paper	-.4269	-.5106	-.8981*	-.9069*

<sup>x</sup>Growth rate was measured at four, eight and 12 d after planting (respectively zero, four and 8 d after inoculation with *S. rolfssii*).  
 “\*” indicates that the correlation coefficient is significant.



**Figure 6.1** Percentage diseased seedlings from 20 *Sclerotium*-inoculated cowpea cultivars using two screening methods (on PDA and on paper) in the laboratory. Seedlings growing on PDA or paper pieces layered in the tube without *Sclerotium*-inoculated twigs were considered as controls. Y error bars are standard deviation values.



**Figure 6. 2** Growth rate (mm/d) of 20 *Sclerotium*-inoculated cowpea cultivars at four, eight and 12 d after planting on PDA and on towel paper. Seedlings growing on PDA or paper pieces layer in the tube without *Sclerotium*-inoculated twigs were considered as controls. Y error bars are Standard deviation values.

## **CHAPTER 7**

# **PHENOLIC CONTENT IN COWPEA AS A SCREENING PARAMETER FOR RESISTANCE OR SUSCEPTIBLE TO DAMPING-OFF AND STEM ROT CAUSED BY *SCLEROTIUM ROLFSII***



**PHENOLIC CONTENT IN COWPEA AS A SCREENING PARAMETER FOR  
RESISTANCE OR SUSCEPTIBLE TO DAMPING-OFF AND STEM ROT  
CAUSED BY *SCLEROTIUM ROLFSII***

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**Abstract**

The role of phenolics in plant resistance to pathogen infection is well documented. The objective of the present investigation was to study possible phenolics involved in resistance or susceptibility of cowpea cultivars to *Sclerotium rolfsii* damping-off and stem rot. Total, free acid, ester-bound and cell wall-bound phenolics were quantified. In healthy seedlings, the tolerant cultivar showed a higher phenol content than the susceptible cultivars. In *S. rolfsii* infected seedlings, the highest increase was found from 48 h after inoculation. The net effect of inoculation was a 630 % increase in total phenolics (soluble and insoluble) in the stem of tolerant cultivars while in the stems of the susceptible cultivars, the total phenolic content increased only by 212 %. Two phytoalexins were detected by thin layer chromatography using toluene/ethyl acetate. No significant difference ( $P = 0.05$ ) was detected among cultivars, in terms of free acid phenolics. However, variability, in terms of ester-bound and cell wall-bound phenolics, was detected among cultivars 48 h after inoculation and the cultivar grouping trend was similar to that of the total phenolics.

Keywords: Cinnamic acids, HPLC, phenolics, phytoalexin, resistance, *Sclerotium*, TLC, *Vigna*.

## 7.1 Introduction

Singh and Rachie (1985) reported that there was virtually no known popular cowpea cultivar with high levels of resistance to the major fungal diseases prevalent in cowpea-production areas in Africa. However, in a field trial in the Ouémé valley, Benin, variation in susceptibility among cowpea cultivars was detected and some were more tolerant than others (PEDUNE-BENIN, unpublished; Adandonon, 2000). Moreover, as shown in screening for resistance to *Sclerotium* damping-off and stem rot (Chapter 6), cowpea cultivars differed in levels of tolerance to the diseases both in the laboratory, greenhouse and field. Resistance mechanism is often characterised by an increase in phenolic compounds or/and production of phytoalexins (Regnier, 1994). This mechanism is not well understood in terms of the host/pathogen interaction between cowpea and *S. rolfsii*.

Several phenolic compounds have been reported to be involved in plant resistance to pests and diseases (Nicholson and Hammerschmidt, 1992; Harborne and Turner, 1984). Singh *et al.* (1997) reported a positive relationship between cowpea resistance/susceptibility characteristics against aphids and qualitative and/or quantitative flavonoid content. Latunde and Lucas (2001) reported that phytoalexins “kievitone” and “paseollidin” accumulated more rapidly in the stem tissue of anthracnose resistant cowpea cultivars. Moreover, it was discovered that dark coloured seeds tend to be more resistant to fungal attack and storage fungi compared to white or cream coloured seeds (Kritzinger, 2000). Recently, it was found that brown cowpea seeds contained a higher concentration of soluble phenolics, mainly flavonoids and were more resistant than white or cream cultivars to *Colletotrichum dematium* (Pers. Ex Fr) Grove (Pakela, 2003). Thus far, no studies have been conducted on the phenolic contents of cowpea and resistance or susceptibility to *S. rolfsii* infection.

The objective of the present work was to study the phenolic compounds deployed during cowpea resistance or susceptibility to *S. rolfsii* damping-off and stem rot.

## 7.2 Materials and Methods

### 7.2.1 Fungal culture

A highly virulent isolate of *Sclerotium rolfsii* from infected cowpea plants in the field in the Ouémé valley, Benin (IITA 408, International Institute of Tropical Agriculture, Cotonou, Benin) was used. Isolate was plated onto potato dextrose agar (PDA) and incubated at 25±1°C. Cultures were maintained on PDA at 4°C.

### 7.2.2 Plant material

Ten cowpea cultivars selected from the results of experiments in Chapter 6 were included in the study. Cultivar characteristics are shown in Table 7.1.

### 7.2.3 Identification of time of maximum total soluble phenolic induction in the cowpea cultivars after *S. rolf sii* infection

#### *Cowpea planting and inoculation*

Two cultivars, namely Tchawé kpayo (susceptible) and Cameroon (tolerant) were used to determine the time of phenolic induction in the stems of plants infected by *S. rolf sii*. Seeds were surface disinfected in 1 % sodium hypochlorite solution for 5 min, rinsed in SDW and planted in pots (14 cm diam. x 18 cm height) at five seeds per pot filled with steam-disinfected sandy loam soil. Five-day old seedlings were inoculated using the inoculum plug method. One 5-mm diameter disc of 4-d-old *S. rolf sii* culture on PDA was cut and placed adjacent to the collar of cowpea seedlings. Wounded seedlings without inoculum plug served as control. Pots were incubated at temperatures varying between 25 to 27 °C and watered every second day.

#### *Preparation of stem samples for extraction*

At 0, 6, 24, 48 and 72 h after inoculation, five seedlings, representing a replicate were uprooted and 6-cm long stem samples were separately cut from the seedling collar upwards. There were three replicates. Samples were frozen in liquid N<sub>2</sub>, ground into a fine powder using a mortar and pestle, and placed in respective Eppendorf tubes for further use.

#### *Extraction and quantification of total soluble phenolics*

The total dry weight (DW) of each of the subsamples (0.05 g) was weighed into 1.5-ml Eppendorf tubes. The samples were separately extracted with 1 ml of methanol/acetone/water (7:7:1 v/v/v) at 4 °C and vortexed in a MixiMatix 90 W vortex (Jencons Scientific Ltd., England) for 1 min. The Eppendorf tubes were placed for 1 h at 4 °C on a rotating shaker (150 rpm) (Labcon 1077 k). Extracts were centrifuged at 10<sup>4</sup> g for 1 min in a microcentrifuge 7200 G (Denver Instrumental Company, USA). The supernatant was retained and three additional extractions were performed with the remaining precipitate as above, in order to extract the maximum soluble phenolic

compounds. The four supernatants were combined and reduced under vacuum and the volume was adjusted to 1 ml with SDW. Samples were stored at 4 °C until further use. The concentration of phenolic compounds was determined using folin-ciocalteau reagent (Swain and Hillis, 1959). The reaction mixture was scaled down to facilitate the use of an ELISA plate. One hundred and seventy microlitres of SDW were dispensed in ELISA plate wells and 10 µl of extract was aliquoted and added, followed by 25 µl of folin ciocalteau's phenol reagent (Sigma) as a colorimetric indicator to each well. Lastly, 50 µl of 20 % (w/v) sodium carbonate was added and gently mixed. A blank of 180 µl of SDW served as a control. The plate was incubated at 40 °C for 30 min. Three wells were used per treatment. Absorbance was read with a Multiskan Ascent V1.24 354-50973 (Version 1.3.1). Gallic acid was used as a phenolic standard to construct a standard curve ranging from 0-400 µg/ml ( $y = 1.3527x - 0.0109$ ,  $r^2 = 0.9989$ ). Phenolic concentration was expressed as gallic acid equivalent per gram dry weight.

#### 7.2.4 Quantification of phenolic compounds (soluble and insoluble) from ten cowpea cultivars at 48 h after inoculation with *S. rolf sii*

In the preliminary experiment to determine the time of maximum phenolic induction in the inoculated cowpea cultivar stem samples after *S. rolf sii* infection, the highest total phenols were recorded from the samples 48 h after inoculation. Therefore, only stem samples at 48 h after inoculation with *S. rolf sii* were evaluated further.

##### *Total soluble phenolics*

Extraction and quantification of the total soluble phenolics were done as described in 7.2.3.

##### *Free phenolic acid*

Tetrafluoroacetic acid (TFA) 20 % (10 µl) was added to a previously prepared sub-sample of seedling stem sample (0.5 ml) in an eppendorf tube. Anhydrous diethyl-ether (0.5 ml) was added; the mixture was then shaken and allowed to stand briefly to get separation of fractions. This process was repeated four times and the separated upper phase layers were removed and combined in another tube. The ether extract was reduced to dryness and the resulting precipitate was resuspended in 0.1 ml methanol. This solution was used to determine the free phenolic acids content with folin-ciocalteau reagent.

*Ester-bound phenolics*

After extraction of the free acids, ammonium sulfate was added to the remaining aqueous phase at the rate of 2 % (w/v) and 0.5 µl of ethyl acetate was added to the remaining part of the aqueous phase. The mixture was shaken and the supernatant was removed as described earlier. The procedure was repeated four times. The supernatants were bulked and reduced to dryness. The resulting precipitate was resuspended in 150 µl of methanol and the amount of methanol soluble ester-bound phenolics was quantified as described in 7.2.3.

*Total insoluble phenolic acids bound to the cell wall*

Sodium hydroxide (0.5 N NaOH) (1 ml) was added to 0.01 g of insoluble residue (sample after methanol:acetone:water, 7:7:1, v/v/v) and sealed in order to prevent oxidation of the free acid produced by saponification. The mixture was left at 96 °C for 1 h. The suspension was hydrolysed by adding 60 µl of concentrated HCl. The process from extraction with anhydrous diethyl-ether to the quantification with the folin-ciocalteu reagent was as described in 7.2.3.

7.2.5 Thin Layer Chromatography (TLC)

To determine the production of phytoalexin in the stem, a one dimensional thin layer chromatography was performed using silica gel plates (10 x 10) and toluene/ethyl acetate (TE) (1:1, v/v) as solvent. All assays were run in duplicate with different volumes loaded to the plate. Spots were visualised with a CAMAT 50H<sub>2</sub> UV camp (254 nm and 366 nm). The visibility of compounds in plates was amplified by flooding the developed plate with liquid nitrogen. The vanillin-HCl test was performed in order to determine the presence of anthocyanidin.

7.2.6 High Performance Liquid Chromatography (HPLC)

HPLC analysis was conducted with a Hewlett Packard 1040 serie II with a normal one-wave length detector (three phases pump). A MALsil C<sub>18</sub>, 5-micron reverse-phase analytical column (250 x 46 mm, 5 µm particle size, chrompack, Separations, Johannesburg) was used together with a precolumn (4.0 mm ID 150 mm long). Samples (20 µl) were analysed with a gradient elution from 7 % solvent A (acetonitrile, HPLC grade), 93 % solvent B (0.1 % aqueous orthophosphoric acid, pH 2.6) to 70 % solvent A

in 45 min. The wavelengths of the detector were 280 and 320 nm. The chromatogram was analysed with a Hewlett Packard HPLC software (Star, Dos series).

### 7.2.7 Statistical analysis

Analysis of variances (ANOVA) was performed on phenolic compound values over the treatments and means separation was done using the Student t-test ( $P = 0.05$ ).

## 7.3 Results

### 7.3.1 Identification of time of maximum total soluble phenolic induction in the cowpea cultivars after *S. rolf sii* infection

The time effect was not significant ( $P = 0.05$ ) for both the control cultivars in terms of the total phenolic content. After inoculation with *S. rolf sii*, the total phenolic content did not differ significantly, especially in the susceptible, until 24 h (Fig. 7.1). In contrast, 48 h after inoculation, the total phenolic content became higher than that recorded 6 and 24 h after inoculation. There was no difference between the total phenolic content, recorded 48 h and 72 h after inoculation with the pathogen. Though the trend was similar for both cultivars, the total phenolic content in the Cameroon cultivar was twice that of the cultivar, Tchawé kpayo.

### 7.3.2 Quantification of phenolic compounds (soluble and insoluble) from the 10 cultivars at 48 h after inoculation with *S. rolf sii*

#### *Total soluble phenolics*

Total phenolic (TP) extract results (Table 7.2) show that cowpea cultivars tested have a pre-existing total phenolic content that is equal to or more than 2.4 mg gallic acid/g DW (or 250 mg gallic acid/100 g DW). The five tolerant cultivars, namely Cameroon, Kpodji, Gboto, Sèwé and Tchawé daho, have significantly higher ( $P < 0.05$ ) pre-existing phenolic content than the susceptible cultivars. The results showed a significant difference ( $P < 0.05$ ) among cultivars in terms of the total phenolic compound present in the stem cell 48 h after inoculation with *S. rolf sii*. Three significantly different ( $P < 0.05$ ) groups were observed in terms of the total phenolic content (Table 7.2): the first group with total phenolic content greater than 19 mg gallic acid equivalent/g Dw were the five tolerant cultivars; the second group with total phenolic content between 14 and 15 mg gallic acid/g DW were IT83D-326-2 and Delekinwa; the third and last group

with total phenolic content less than 10 mg gallic acid equivalent/g DW consisted of the susceptible cultivars namely TVU 72-74, K VX-61-1 and Tchawé kpayo.

#### *Free phenolic acid*

The free acid content from the tested cultivars was between 0.4 and 0.7 when cultivars were not inoculated (Table 7.3). When inoculated, cultivars showed free phenolic acid values that were higher than when uninoculated, although not significantly ( $P = 0.05$ ). After inoculation, the free phenolic acid represented a proportion less than 10 % of the total phenolic compound content in the cell (Table 7.3).

#### *Ester-bound phenolics*

Forty-eight hours after inoculation, the tolerant cultivars had the highest ester-bound phenolic values, however, representing less than 20 % of the total phenolic compound content in the cell (Table 7.3).

#### *Insoluble phenolic acids bound to the cell wall*

The cell wall-bound phenolics represented more than 70 % of the total phenolic compounds in the cell at 48 h after inoculation with *S. rolf sii* (Table 7.3). There was a significant difference among cultivars and the highest values (more than 14 mg gallic acid/g DW) were recorded with the tolerant cultivars. Moreover, the cell wall-bound phenolic values for the tolerant cultivars at 48 h after inoculation were more than eight-fold the content value when cultivars were not inoculated (Table 7.3).

### 7.3.3 Thin Layer Chromatography and High Performance Liquid Chromatography analysis

TLC and HPLC analyses were performed only for the crude extracts. The TLC showed a strong blue fluorescence under UV light, indicating that hydroxycinnamic acid might be present. Four fluorescent bands [a (rf = 0.24), b (rf = 0.53), c (rf = 0.74) and d (rf = 0.99)] (Fig. 7.2) were visible, under UV light, for the *Sclerotium*-inoculated tolerant cultivars. In the intermediate inoculated cultivars, all bands were observed, but the fluorescence of the spot c was low. Only two bands (a and b) were observed in the inoculated Tchawé kpayo (susceptible) cultivar. In the uninoculated (control) cultivars, spots were visible only at band b for most of the cultivars. No apparent spot was found in the uninoculated (control) Tchawé kpayo for all the concentration tested. Vanillin HCl tested negative, indicating that anthocyanidin was absent. Analytical HPLC

chromatogram showed five peaks [1 (rt = 12.5 min); 2 (rt = 17 min), 3 (rt = 34 min), 4 (rt = 33.6 min) and 5 (rt = 37.5 min)] at 320 nm corresponding to five compounds in the inoculated Cameroon cultivar samples (Fig 7.3A) (with a strong peak) that were absent in the uninoculated samples (Fig. 7.3A and B). No difference was found between control and inoculated samples at 280 nm.

#### 7.4 Discussion

The role of phenolic compounds in the host/pathogen interaction is well established (Sarma *et al.*, 2002) and constitutive phenolics are known to confer resistance indirectly through activation of post-infection responses in the host (Harborne, 1988). Several studies have shown that some phenolics are inhibitors associated with non-host resistance (Nicholson and Hammerschmidt, 1992) whereas others are formed or increased in response to pathogen infection, and are considered to be an important component in the defence response of the host to the pathogen (Harborne and Turner, 1984; Punja *et al.*, 1985; Nicholson and Hammerschmidt, 1992).

Results in the present study showed that the total phenolic compound content in the seedling stems, before inoculation with *S. rolf sii*, differed significantly ( $P < 0.05$ ) among cultivars. Bouchereau *et al.* (1992) indicated that plant phenolics provide metabolic functions during the seedling and vegetative growth. However, according to Poorter and Bergkotte (1992), the production of phenolics as well as their stability can be influenced by several factors such as light, temperature, pH, and substrate conditions. In our study, these parameters were similar for all cultivars indicating that the content of soluble phenolics could represent a good parameter for screening cowpea for resistance to *S. rolf sii* infection. Koricheva *et al.* (1998) reported that the allocation of carbon through a hierarchical level of reaction is mainly directed by the plant needs in terms of growth versus defences. In cowpea, all the cultivars tested showed the same growth habit. This supports the idea that the tolerant cultivars at the seedling stage are able to allocate their carbon to the defence system better than the susceptible ones.

The inoculation study showed that the plants recognised the pathogen 6 h after the inoculation. However, significant difference was detected between control and inoculated only 24 h and 48 h after the inoculation for tolerant and susceptible cultivar, respectively. Similar results were found in tomato after inoculation with *Clavibacter* sp. (Matta *et al.*, 1988; Beimen *et al.*, 1992). A strong relationship was detected between total phenol content and the tolerance/susceptibility status of the cultivars, the more



tolerant the cultivars the higher the phenolic content after inoculation. The TLC analysis showed compounds from the hydroxycinnamic family are present. Furthermore, no anthocyanidin could be detected with the vanillin-HCl. These results could indicate that, when *S. rolf sii* infects cowpea, several esters, especially cinnamic acids, are induced in the tolerant cultivars, which are able to withstand the infection caused by the pathogen. In tomato leaves, Beimen *et al.* (1992) reported that a pathogen-induced time-dependent increase in phenolics such as chlorogenic and cell wall-bound cinnamic acids occurred in response to *Clavibacter* spp. inoculation. In chickpea (*Cicer arietinum* L.), Sarma *et al.* (2002) also reported that phenolics including gallic, ferulic, chlorogenic and cinnamic acids were present in leaves, collar and roots of the plant and the concentration increased when plants were inoculated with *S. rolf sii*. The authors indicated that the highest phenolics were induced when plants were inoculated with *S. rolf sii* and treated with rhizobacteria, and found a direct relationship between the level of total phenolic content and seedling tolerance. The relationship between phenolics and resistance of the plant was also reported in cowpea leaves by Lattanzio *et al.* (1997) and in bean leaves by Nozzolillo *et al.* (1998). To our knowledge, no research has been done linking content of phenolics in the stem of cowpea to resistance to *S. rolf sii* and our study appears to be the first report on the subject. The production of two compounds (c and d) were observed by the TLC analysis in all the inoculated cultivars and in one susceptible and one intermediate cultivars. The HPLC analysis confirmed the TLC results. Two phytoalexins could then be produced by the cowpea seedlings in response to *S. rolf sii* infection. This result is similar to that found by Latunde and Lucas (2001). The authors reported that two types of phytoalexins, namely “kievitone” and “paseollidin”, accumulated more rapidly in the stem tissue of anthracnose resistant cowpea cultivars. Phytoalexins were reported in early studies on the Solanaceae (as terpenoids) and in most legumes (as isoflavonoids) (Kuć, 1995; Singh *et al.*, 1995). Phytoalexins produced in plants could accumulate around both resistant and susceptible necrotic tissues (Kuć, 1995). In the case of susceptible cowpea cultivars, the production of a small amount of phytoalexins 48 h after inoculation appears to be the first attempt to stop the pathogen. However, the concentration of these phytoalexins is not sufficient to be toxic and therefore to prevent the growth of the pathogen. Nicholson and Hammerschmidt (1992) and Kuć (1995) indicated that resistance occurs in plant when phytoalexins reach a concentration sufficient to restrict pathogen development. Moreover, Bailey and Mansfield (1982) reported that formation of phytoalexins in a

susceptible host following infection by a pathogen seems to be prevented by suppressor molecules produced by the pathogen. Results found with the tolerant and susceptible cultivars tested in the current study might confirm these early reports.

The content of soluble free acid found in the stem did not vary over cultivars, though it increased 48 h after inoculation. The low percentage (10 % of the total pool of phenolic compounds) found in the stem is similar to previous data reported in wheat (*Triticum turgidum* L.) (Regnier, 1994). The low increase of free acid content in the stem after inoculation by the pathogen is not unusual and can be explained by the orientation of the metabolism to the esterification of these acids and further to an integration of the phenolics into the cell wall in order to protect the plant cells against the invasion (Fry, 1979; 1983).

After inoculation, the ester-bound and cell wall-bound phenolics were induced more than 1.5- and 8-fold, respectively, compared to the controls. These data are consistent with early reports on other crops (Matta *et al.*, 1988; Regnier, 1994). The first contact of a pathogen with a plant occurs at a plant surface or cell walls that produce, modify and accumulate several defence-related substances, such as phenolic compounds, and reinforce the resistance of the cell wall to invasion by the pathogen (Dixon *et al.*, 1994). As part of the lignin product, the ferrulic and hydroxycinnamic acid are usually the main acid esterified into the cell wall. In a susceptible case, the host fails to produce reinforcing compounds or produces them too slowly to be effective, and the fungus manages to invade the cell (Nicholson and Hammerschmidt, 1992; Dixon *et al.*, 1994; Kuć, 1995). The results in the present study showed that the highest cell wall-bound phenolics were produced in the tolerant cultivars and the lowest in the susceptible ones. It shows that phenolics present before and after *S. rolfsii* infection, could be used as screening parameters for resistance or tolerance to *S. rolfsii* damping-off and stem rot.

The results found in the current study show that phenolic compound, main ester-bound soluble and cell wall-bound phenolics are the major phenolics in cowpea cultivars involved in the resistance or tolerance of the plant against damping-off and stem of cowpea caused by *S. rolfsii*. However, further research needs to be done, especially on the characterisation and purification of the phytoalexins involved in the tolerance.

## 7.5 Acknowledgements

This work was financed by the National Research Foundation, South Africa. Thanks are due to Wilma du plooy for HPLC graphs

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**Table 7.1**

Characteristics of 20 cowpea cultivars used during the phenolic studies

Cultivars	Colour	Origin	Status <sup>x</sup>
Cameroon	Red-brown	Cameroon Benin local	Tolerant
Kpodji	Black	Benin local	“
Gboto	Red	Benin local	“
Sèwé	Black	Benin local	“
Tchawé daho	White	IITA	“
IT89D-326-2	White	Benin local	Intermediate
Délékinwa	Cream	Nigeria	“
TVU72-74	White	Burkina Faso	Susceptible
KVX-61-1	White with cream spot	Benin local	“
Tchawé kpayo	White		“

<sup>x</sup>Characteristics based on Chapter 6 results.

**Table 7.2**Total phenolic compounds in the cells 48 h after inoculation with *S. rolf sii*

Cultivars	Total phenolic compounds <sup>1</sup> (mg gallic acid/g Dw)	
	Control	Inoculated
Cameroon	3.6 ± 0.06 e	26.3 ± 0.13 d
Kpodji	3.2 ± 0.07 c	19.8 ± 0.07 c
Gboto	3.5 ± 0.02 de	19.4 ± 0.07 c
Sèwé	3.3 ± 0.07 cd	20.4 ± 0.08 c
Tchawé daxo	3.1 ± 0.07 bc	19.4 ± 0.08 c
IT83D-326	2.9 ± 0.05 b	14.1 ± 0.09 b
Delekinwa	3 ± 0.07 b	14.3 ± 0.12 b
TVU72-74	2.6 ± 0.09 a	8.2 ± 0.06 a
KVX61-1	2.4 ± 0.06 a	8.4 ± 0.11 a
Tchawé kpayo	2.5 ± 0.06 a	7.8 ± 0.04 a

<sup>1</sup>Values are followed by the standard error. Within a column, values not followed by the same letters are significantly different ( $P < 0.05$ ) according to Student t-test.

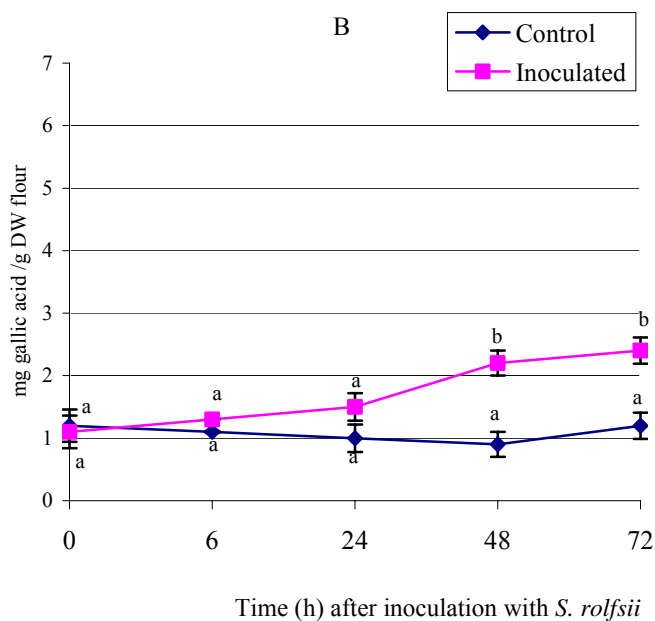
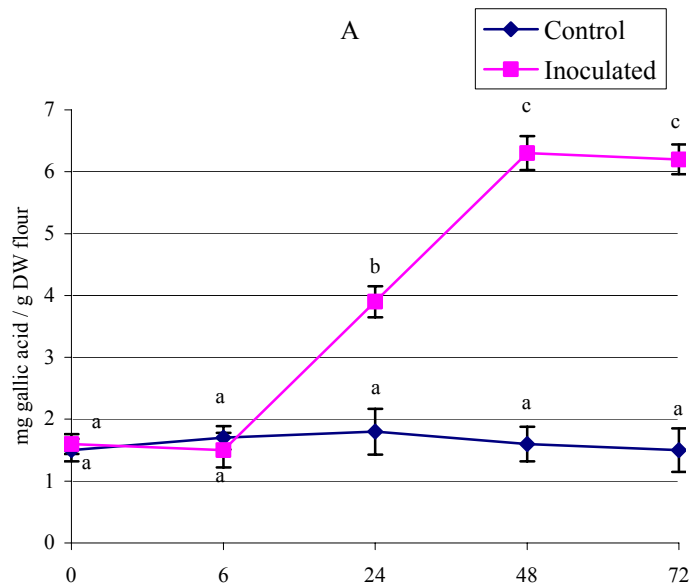
**Table 7.3**

Total soluble and insoluble phenolics, and their percentage compared to the total phenolic compounds in the cells 48 h after inoculation with *S. rolfsii*

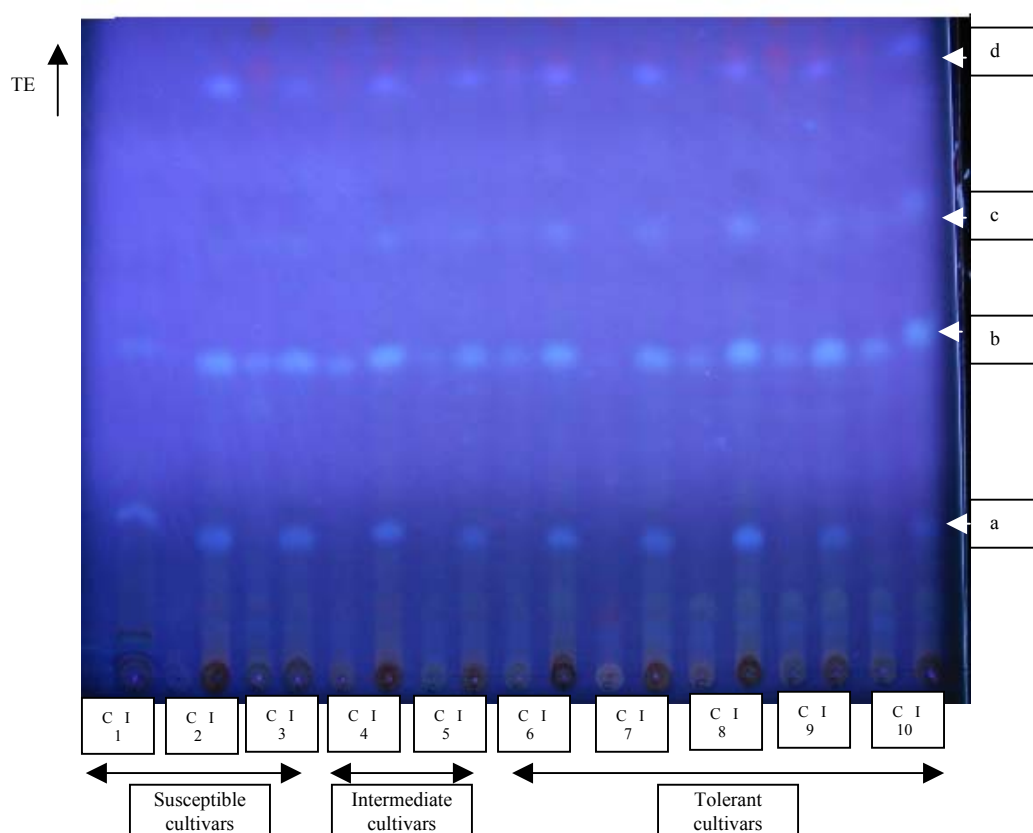
Cultivars <sup>1</sup>	Total soluble phenolics <sup>2</sup> (mg gallic acid/g Dw)				Insoluble phenolics <sup>2</sup> (mg gallic acid/g Dw)	
	Free acid		Ester-bound		Cell wall	
	Control	inoculated	Control	Inoculated	Control	Inoculated
Cameroon	0.7 ± 0.07 a	1.6 ± 0.29 a (6.1 %)	1.2 ± 0.31 a	4.3 ± 0.28 e (16.3 %)	1.6 ± 0.13 e	20.4 ± 0.18 d (77.6 %)
Kpodji	0.6 ± 0.06 a	1.5 ± 0.28 a (7.6 %)	1.1 ± 0.14 a	3.7 ± 0.11 d (18.7 %)	1.5 ± 0.04 de	14.6 ± 0.31 c (73.7 %)
Gboto	0.4 ± 0.11 a	1.1 ± 0.09 a (5.7 %)	0.9 ± 0.05 a	2.9 ± 0.07 c (14.9 %)	1.6 ± 0.14 e	15.4 ± 0.24 c (79.4 %)
Sèwé	0.7 ± 0.34 a	1.0 ± 0.04 a (4.9 %)	0.8 ± 0.09 a	3.1 ± 0.04 c (15.2 %)	1.3 ± 0.13 bc	16.3 ± 0.12 c (79.9 %)
Tcha. daxo	0.8 ± 0.13 a	1.3 ± 0.25 a (6.7 %)	1.1 ± 0.20 a	3.2 ± 0.19 c (16.5 %)	1.4 ± 0.19 cd	14.9 ± 0.17 c (76.8 %)
IT83D-326	0.7 ± 0.16 a	1.1 ± 0.05 a (7.8 %)	0.9 ± 0.11 a	2.3 ± 0.10 b (16.3 %)	1.3 ± 0.08 cd	10.7 ± 0.36 b (75.9 %)
Delekinwa	0.6 ± 0.09 a	1.2 ± 0.22 a (8.4 %)	1.1 ± 0.15 a	2.2 ± 0.23 b (15.4 %)	1.2 ± 0.12 b	10.9 ± 0.01 b (76.2 %)
TVU72-74	0.5 ± 0.06 a	0.7 ± 0.17 a (8.5 %)	1.0 ± 0.19 a	1.7 ± 0.04 a (20.7 %)	1.0 ± 0.12 a	5.8 ± 0.09 a (70.7 %)
KVX61-1	0.7 ± 0.03 a	0.8 ± 0.02 a (9.5 %)	1.0 ± 0.24 a	1.4 ± 0.32 a (16.7 %)	1.0 ± 0.06 a	6.2 ± 0.09 a (73.8 %)
Tcha. Kpayo	0.4 ± 0.09 a	0.8 ± 0.11 a (9.0 %)	0.8 ± 0.07 a	1.3 ± 0.22 a (16.7 %)	0.9 ± 0.20 a	5.7 ± 0.32 a (73.1 %)

<sup>1</sup>Tcha. Kpayo = Tchawé kpayo; Tcha. Daho = Tchawé daho. <sup>2</sup>Values are followed by the standard error. Within a column, values not followed by the same letters are significantly different ( $P < 0.05$ ) according to Student t-test. Values in parenthesis within a row express the percentage of the phenolics compared to the total phenolic compounds in the stem cell.

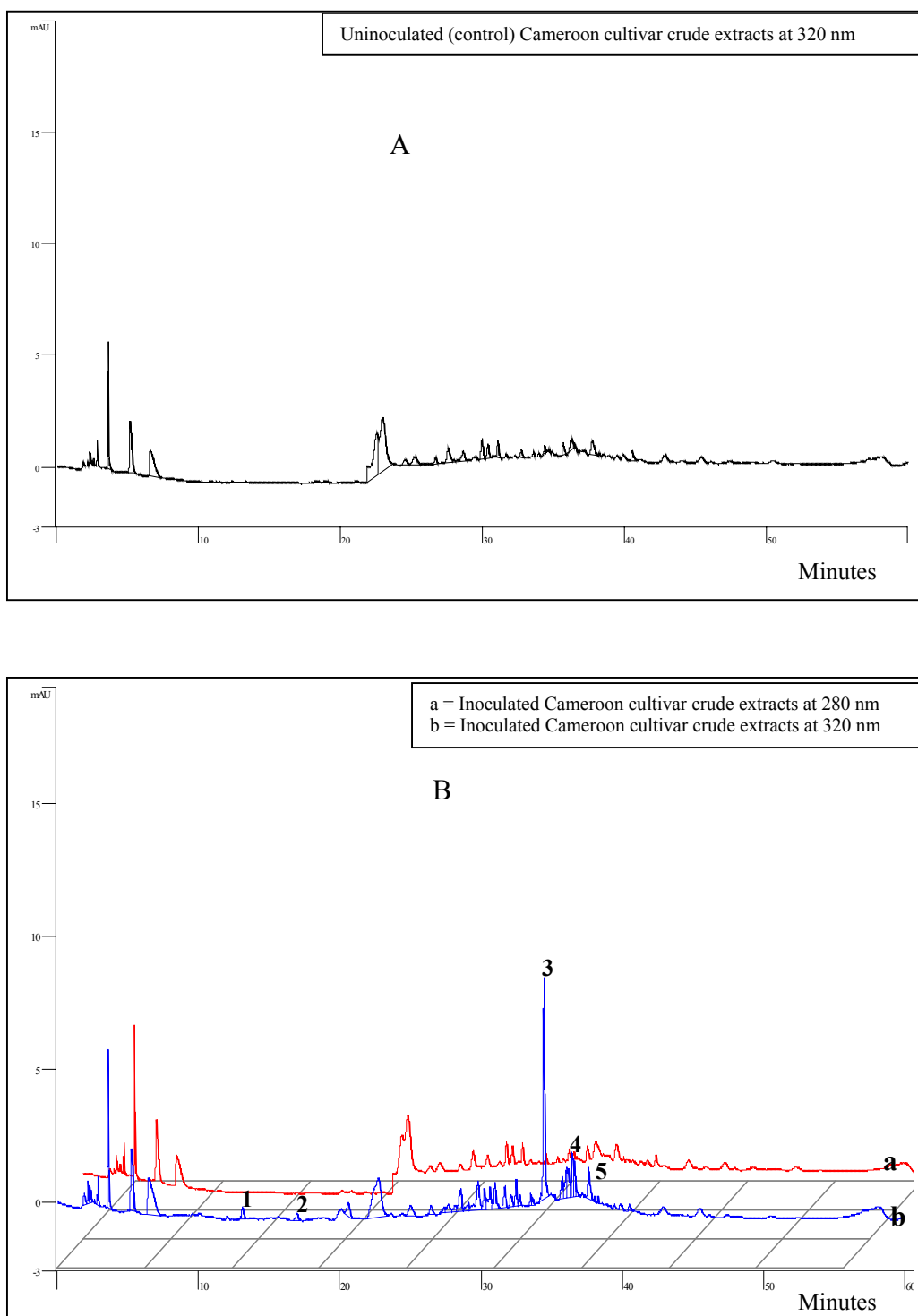




**Figure 7.1** Effect of time (h) after *S. rolf sii* infection, on maximum total soluble phenolic induction in two cowpea cultivars: a tolerant, Cameroon (A); and a susceptible, Tchawé kpayo (B). Five-day old seedlings were inoculated using inoculum plug (of 4-day old culture of *S. rolf sii* on PDA) placed adjacent to each seedling collar. Total soluble phenolics were determined at 0, 6, 24, 48 and 72 h after inoculation. Controls were uninoculated seedlings.



**Figure 7.2** Plate produced on toluene/ethyl acetate solvent, showing four bands [a (rf = 0.24), b (rf = 0.53), c (rf = 0.74) and d (rf = 0.99)] that were present in the crude extracts of ten cowpea cultivars. TE = toluene/ethyl acetate solvent. Number 1 to 10 are cultivars: 1 = Tchawé kpayo; 2 = K VX62-1; 3 = TVU72-74; 4 = Delekinwa; 5 = IT83D-326-2; 6 = Tchawé daho; 7 = Sèwé; 8 = Gboto; 9 = Kpodji and 10 = Cameroon; C = uninoculated (control) and I = inoculated with *S. rolf sii*.



**Figure 7.3** HPLC analysis of a crude extract of a cowpea cultivar (Cameroon) 48 h after inoculation with *S. rolf sii*: Profile of the control sample at 280 nm (A), profile of the inoculated sample analysed at 280 and 320 nm (B). The graph b) showed five peaks that were absent in graph a): 1 (rt = 12.5 min); 2 (rt = 17 min), 3 (rt = 34 min), 4 (rt = 33.6 min) and 5 (rt = 37.5 min)].

## **CHAPTER 8**

# **EFFICACY OF *MORINGA OLEIFERA* LEAF EXTRACTS AGAINST *SCLEROTIUM ROLFSII* AND DAMPING-OFF AND STEM ROT OF COWPEA**

**EFFICACY OF *MORINGA OLEIFERA* LEAF EXTRACTS AGAINST *SCLEROTIUM ROLFSII* AND DAMPING-OFF AND STEM ROT OF COWPEA**

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**Abstract**

Seedling damping-off and stem rot are harmful to cultivated plants in moist tropics and in warm temperate areas. Laboratory, greenhouse and field trials were conducted to test the effectiveness of *Moringa oleifera* L. leaf extracts on cowpea damping-off and stem rot caused by *Sclerotium rolfsii*. In the laboratory test, *M. oleifera* leaf extract was mixed with kerosene and amended to PDA, and mycelial growth of *S. rolfsii* measured. In the greenhouse and field experiments, cowpea seeds were soaked in *M. oleifera* extracts mixed with both kerosene and soap before planting. Percentage disease incidence, severity and control were recorded. In the laboratory, the higher the extract concentration the less the mycelium growth and no colony mycelium was formed on PDA amended with extract at 15 g/10 ml sterile distilled water. There was no significant difference ( $P = 0.05$ ) between extract at 15 and 20g/10 ml distilled water. In the greenhouse, a significant effect of the application of *M. oleifera* leaf extracts was observed on *Sclerotium* damping-off and stem rot of cowpea. The highest disease control using the extract was obtained at a concentration of 15 kg/10 l distilled water (w/v) with no significant difference ( $P = 0.05$ ) between concentrations of 15 and 20 kg/10 l solution. The disease control of the extracts in the field experiment followed a similar trend to but was less than that observed in the greenhouse. However, this is the first report showing the effectiveness of *M. oleifera* leaf extract (15 kg/10 l distilled water, w/v) mixed with kerosene (1 % v/v) and soap (0.1 % w/v) in the control of *S. rolfsii* on cowpea both in the greenhouse and field.

Keywords: Biopesticide, soilborne fungi.

## 8.1 Introduction

Damping-off and stem rot of cowpea, *Vigna unguiculata* [L.] Walp, caused by *Sclerotium rolfsii* Sacc. has been reported in many countries (Singh *et al.*, 1997; Adandonon, 2000; Kossou *et al.*, 2001) and in the Ouémé valley in Benin, the diseases are a major constraint to the production of cowpea (PEDUNE-BENIN, 1995). Control of *Sclerotium* diseases may be achieved by cultural means (Singh and Allen, 1979) or by using chemical fertilisers such as ammonium-types of calcium compounds. In some cases, fungicides such as pentachloronitrobenzene (PCNB), captafol, and dichloran are applied to the seeds or to the soil before planting or in furrows during planting (Punja, 1985). Chloroneb and prothiocarb can be used as soil drenches or as in-furrow sprays near the roots of crops (Jones, 1987). In Benin, the only registered fungicide used on edible crops such as cowpea is Super-Homai 70 % PM (active ingredient: methylthiophanate 35 %, thiram 20 %, and diazinon 15 %) (SPV, Benin). Unfortunately, there is still a problem with its effectiveness (Kakpo-Zannou, B.L., Plant Protection Services, Porto-Novo, Benin, Pers. Comm.). Despite the effectiveness of fungicides, the widespread use of chemicals has become a subject of public concern, mainly due to their potential harmful effect on non-target organisms, the development of resistant races of pathogens, and the possible carcinogenicity of some chemicals (Zaki *et al.*, 1998). Other problems include gradual elimination and phasing out of some available pesticides, and the reluctance of some chemical companies to develop and test new chemicals due to escalating development and registration costs (Zaki *et al.*, 1998). Thus, there is a need to examine the potential for non-synthetic chemical approaches to disease management.

Singh *et al.* (1980) indicated that some plant organs have fungicidal properties and plant extracts have effectively been tested in the control of some soil-borne pathogen diseases such as *Rhizoctonia* and *Pythium* rot diseases. Similarly, Stoll (1988) reported the fungicidal seed treatment effect of *M. oleifera* leaf extract on some soilborne fungi in cereals. Unfortunately, plant extract active ingredients were reported to easily breakdown, thereby reducing the persistence. Any additive that helps to slow down the ingredient degradation would improve the efficacy of these extracts. Kerosene was reported to have properties that improve extraction of active ingredients from plant organs and reduce the degradation thereof (Stoll, 1988). It has effectively been used with some plant extracts (Stoll, 1988). However, no report exists on the use of *M. oleifera* extract and the appropriate dose of kerosene to be added in the control of *S. rolfsii* causing damping-off and stem rot of cowpea.

The aim of this study was to evaluate *M. oleifera* leaf extracts for their efficacy in the control of *S. rolfsii* on PDA and damping-off and stem rot of cowpea in the greenhouse and field.

## 8.2 Materials and Methods

### 8.2.1 Pathogen culture

A highly virulent *S. rolfsii* isolate (IITA 409) collected from infected cowpea plants in the Ouémé valley, Benin, was used. The isolate was sub-cultured and maintained on potato dextrose agar (PDA) slants at 4 °C.

### 8.2.2 *In vitro* effect of *Moringa oleifera* leaf extracts on mycelial growth of *S. rolfsii*

#### *Preparation of Moringa oleifera leaf extracts*

Fresh *M. oleifera* leaves were collected in Abomey-Calavi, Benin (in July and November 2002 and 2003) and 5, 10, 15 and 20 g samples were weighed out and surface sterilised for 2 min in 70 % ethanol. Leaves were then rinsed twice in sterile distilled water (SDW) and separately crushed into a pulp using sterilised porcelain mortars and pestles. The pulp of each sample was suspended in 10 ml of SDW.

Three different volumes of kerosene, namely 0.1, 0.2 and 0.3 ml were added to 10 ml SDW to yield final concentrations of 1, 2 and 3 % v/v. Each concentration was separately added to each of the four extract suspensions. The extract-kerosene suspensions were agitated for 2 min and stored overnight at 26-28 °C and then filtered through sterile cotton cloth into another sterile Erlenmeyer flask to yield the final extract.

#### *Effect of Moringa oleifera leaf extracts on S. rolfsii growth*

Thirty millilitres of the sterile extract concentration was added to 300 ml cooled potato dextrose agar (PDA) amended with chloramphenicol (25 mg/l) and carefully agitated to allow for proper mixing of extract and media. Twenty-millilitre aliquots of the amended media were dispensed into 90-mm Petri dishes. This extract proportion of 30 ml used was based on a preliminary study (Adandonon, unpublished).

Once the amended agar had solidified, 2-mm discs cut from the actively growing edge of a 3-d-old colony of *S. rolfsii* on PDA was placed in the centre of each plate. The controls consisted of the pathogen grown on unamended PDA and PDA-amended kerosene. There were four plates per replicate per concentration and five replicates.

Plates were incubated at  $25 \pm 1$  °C and the radial growth of the colonies was measured after 5 d of incubation.

### 8.2.3 *In vivo* effect of *Moringa oleifera* leaf extracts on damping-off and stem rot incidence in the greenhouse

#### *Moringa oleifera* extract preparation with kerosene and soap

In the greenhouse experiment, 0.5, 1.0, 1.5 and 2.0 kg of *M. oleifera* leaves were weighed, ground and the pulp was suspended in 1 l of SDW as described earlier. These quantities were in the same proportions as those used in the *in vitro* experiment. In the *in vitro* experiment, results (Table 8.1) showed no significant difference in colony growth among the three different doses of kerosene (plus *M. oleifera* extracts) tested. Therefore, only 1 % kerosene (v/v) was evaluated. Treatments included: extract combined with kerosene (1 % v/v) and Marseilles soap (0.1 % w/v), extract with 1 % kerosene only, extract with 0.1 % soap only, extract alone, 1 % kerosene alone and 0.1 % soap alone. The use and quantity of Marseilles soap in this experiment was based on earlier study results on other plants (Stoll, 1988). The kerosene and the soap were added before the suspension was stored overnight, as described earlier. Super-Homai 70 % PM (active ingredient: methylthiophanate 35 %, thiram 20 %, and diazinon 15 %), a registered fungicide in Benin, was considered the positive control, whereas SDW alone and no treatment were the negative controls.

#### *Soil inoculation with the pathogen, seed treatment and planting*

Sandy loam soil from the experimental farm of the International Institute of Tropical Agriculture (IITA, Benin) was steam disinfected and stored for 21 d before inoculation (Pieczarka and Abawi, 1978). Soil inoculation with *S. rolfsii* using the millet seed inoculation technique (Weideman and Wehner, 1993) was done 3 d before planting in the greenhouse. Ten grams of the *S. rolfsii* millet seed inoculum was mixed with 1 kg soil in a pot (14 cm diam. x 18 cm height).

Seeds of two different damping-off- and stem rot-susceptible cowpea cultivars, namely Tchawé kpayo and IT89KD-374-57 were used. Before planting, seeds were soaked for 5 min (PEDUNE-BENIN, 1995) in the different extract suspensions. The seeds were planted at four seeds per pot. Three pots represented a treatment. The experimental design was a randomised block with four replicates. Pots were maintained in the greenhouse at temperature varying between 26-30 °C and relative humidity between 65-90 %. Pots were watered daily in the morning.



#### *Data collection*

The number of damping-off seedlings and stem rot plants were recorded 4 d after planting and at 2-d intervals until 30 d after planting. The disease severity per plant was determined based on a scale of 0 to 6 (Adandonon, 2000): 0-: No visible symptoms; 1-: Leaves wilted; seedlings fell over after the 4<sup>th</sup> d; 2-: Leaves wilted on 3<sup>rd</sup> d; plants fell over on 4<sup>th</sup> d; 3-: Leaves wilted on 3<sup>rd</sup> d; plants fell over on the ground on 3<sup>rd</sup> d; 4-: Leaves wilted on 2<sup>nd</sup> d; plants fell over on 2<sup>nd</sup> or 3<sup>rd</sup> d; 5-: Leaves wilted on 1<sup>st</sup> d; plants fell over on 2<sup>nd</sup> d and; 6-: Leaves wilted; plants fell over within 24 h.

#### 8.2.4 *In vivo* effect of *Moringa oleifera* leaf extracts on damping-off and stem rot incidence in the field

The trial was conducted during two seasons (2002 and 2003) in the Ouémé valley in fields naturally infected with *S. rolfsii*. The variation in both recorded climate parameters was higher in 2002 than in 2003. All treatments in the greenhouse were also tested in the field experiment. The experimental design was a randomised block design with two controls and four replicates. Control 1: untreated seeds; Control 2: cowpea seeds treated with fungicide (Super-Homai) before planting. A 200-m<sup>2</sup> Plot was assigned to each treatment and was planted with the respective treated cowpea seeds. Row spacing, number of seeds per row and other cultural practices were those of farmers in the valley.

The number of plants present 7 d after planting was counted. From 7 d after planting onwards, the number of damping-off seedlings and stem rot plants was recorded as disease incidence at 2-d intervals until 30 d after planting. The percentage disease control in each treatment was calculated as follows:

$$DR \% = [1 - (DT / DC)] * 100$$

where DR : Disease control or reduction;

DT : Disease incidence in the treatment plot and

DC : Disease incidence in the control plot (untreated).

#### 8.2.5 Statistical analysis

The statistical analysis was performed as described in Chapter 3.

### 8.3 Results

#### 7.3.1 *In vitro* effect of *Moringa oleifera* leaf extracts on the mycelial growth of *S. rolfsii*

The mean fungal radial growth (mm), recorded for each treatment on PDA is presented in Table 8.1. In all cases where PDA was amended with the extract alone or mixed with kerosene, it resulted in significantly less mycelial growth than that of kerosene alone-amended PDA or unamended PDA (control) (Table 8.1). Extracts combined with kerosene, showed less mycelial growth than did respective extracts alone. At a given extract concentration, there was no significant difference among the kerosene doses. The effect of extract was more pronounced at higher concentrations: the higher the extract concentration the less the mycelial growth and no colony mycelium was formed on PDA amended with extracts at 15 and 20 g/10 ml. There was no significant difference ( $P = 0.05$ ) between extracts at 15 and 20 g/10 ml (Table 8.1).

#### 8.3.2 *In vivo* effect of *Moringa oleifera* leaf extracts on the *Sclerotium* damping-off and stem rot expression in the greenhouse and field experiment

In the greenhouse, the disease incidence recorded with the extracts, alone or mixed with kerosene and soap, regardless of extract concentration was the least, compared to either control, kerosene alone, soap alone, water or Super-Homai.

At a given concentration, extract mixed with both kerosene and Marseilles soap yielded disease incidences less than that of extract mixed with either kerosene or soap. No or less than 0.1 % disease incidence was recorded with an extract concentration of 15 kg/10 l mixed with kerosene and soap. There was no significant difference ( $P = 0.05$ ) between the concentrations 15 and 20 kg/10 l in terms of percentage disease incidence. The treatment effect trend in terms of disease incidence was similar for both cowpea cultivars used (Table 8.2). The lowest disease severity was recorded, in both cowpea cultivars, when seeds were treated with *M. oleifera* extract concentration of 15 or 20 kg/10 l water combined with kerosene and soap (Table 8.2).

The air temperature and relative humidity recorded in the field between 08h00 and 14h00 varied between 25.7 to 27.8 °C and 62 to 97 %, respectively in 2002, and 25.8 to 27.4 and 65 to 76 %, respectively in 2003. The trend in disease incidence for the treatments in the field (Table 8.3) was similar to that recorded in the greenhouse. However, the percentage disease control in the field trials when compared to greenhouse experiments (Table 8.3) varied significantly ( $P < 0.05$ ). In the greenhouse

the disease control recorded with 15 or 20 kg/10 l was more than 98 %, while the disease control for both concentrations in the field was less than 71 %.

#### 8.4 Discussion

The potential of plant extracts to control soilborne diseases has long been recognised (Ark and Thompson, 1959). Results from the present study show a significant effect of the *M. oleifera* leaf extracts on *Sclerotium* growth on PDA. No growth was recorded with an extract concentration of 15 or 20 kg/10 l solution. These results indicate that the treatment affected the mycelial growth and any further development of the pathogen. This confirms its antifungal activities as reported earlier (Stoll, 1988; Garcia and Garcia, 1990). SIBAT (1993) reported that *M. oleifera* leaf extracts inhibited the *in vitro* growth of some fungal pathogens such as *Pythium* sp., causal agent of damping-off of legumes and vegetables. Plant extract effects on pathogen growth on PDA are well documented (Garcia and Garcia, 1990; Obagwu *et al.*, 1997; Obagwu and Korsten, 2003). However, this is the first report of effect of the *M. oleifera* extract on *S. rolfsii* mycelium growth on PDA in the laboratory.

In the greenhouse, significant disease control was recorded when *M. oleifera* extracts of 15 or 20 kg/10 l were mixed with kerosene and soap, indicating that a concentration of 15 kg/10 l of water is enough for adequate *Sclerotium* disease control in the greenhouse. In the field, the trend was similar, and the effect of the *M. oleifera* extracts on the disease control was the highest at the higher concentrations. However, the disease control in the field was less than that in the greenhouse. Results indicated that a *M. oleifera* extract concentration of 15 kg/10 l of water was enough for adequate *Sclerotium* disease control in the field. Probably large soil volume and leaching effect in the field was the cause of lower efficacy. The two cowpea varieties used in the experiment showed the same trend for all treatments. Extracts from plants such as garlic (*Allium sativum* L.) (Russell and Musa, 1977; Garcia and Garcia, 1990; Obagwu and Korsten, 2003), neem (*Azadirachta indica* Juss) and pawpaw (*Carica papaya* L.) (Stoll, 1988) have been tested on many soilborne fungi. There are, however, few references on the use of *M. oleifera* extracts to control plant pathogens. Stoll (1988) reported the fungicidal effect of *M. oleifera* leaf extracts on some soilborne fungi such as *Rhizoctonia*, *Pythium* and *Fusarium* in cereal crops. In the current study, the *M. oleifera* extracts also showed a biologically significant effect on *S. rolfsii*. The extract-kerosene-soap mixture effect was higher than that of extracts alone or mixed with either kerosene

or soap. Super-Homai was the fungicide used in this study as a positive control, but was not as effective, when compared to the *M. oleifera* extracts.

This proven fungicidal effect of *M. oleifera* extracts in the field is quite promising since biological or plant based-product controls offer durable, safe and cost-effective alternatives to soil-applied chemicals (Hornby, 1990). *Moringa oleifera* is native to India but has been planted and naturalised in many areas around the world (Davis, 2000). Furthermore, a previous study showed that its leaves are relatively easy to crush for extraction, compared to papaw and neem (Adandonon, unpublished).

This is the first report to show the effectiveness of *M. oleifera* leaf extract in the control of *S. rolfsii* on cowpea both in the greenhouse and field. Mechanisms of action of the extracts are not well understood. However, *M. oleifera* leaves contain some crystalline alkaloids (SIBAT, 1993), fatty acid, proteins, and glycosides said to be responsible for the antimicrobial activities (<http://www.essentialdrugs.org/emed/archive>). Further work will aim to increase the efficacy of the extracts in the field and determine the main biologically active ingredient of *M. oleifera* extract and its mode of action.

### 8.5 Acknowledgements

This work was financed by the International Institute of Tropical Agriculture (IITA), Ibadan, Nigeria. We are grateful to Mr Sam Korie for data statistical analysis.

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**Table 8.1**

*In vitro* effect of *Moringa oleifera* leaf extract and kerosene on the mycelial growth of *Sclerotium rolfsii* on PDA

Treatments <sup>1</sup>	Colony diameter (mm) <sup>2</sup>
<i>Moringa</i> 5 + K1%	33.6 c
<i>Moringa</i> 5 + K2%	36.5 c
<i>Moringa</i> 5 + K3%	38.7 c
<i>Moringa</i> 10 + K1%	14.4 b
<i>Moringa</i> 10 + K2%	17.8 b
<i>Moringa</i> 10 + K3%	12.3 b
<i>Moringa</i> 15 + K1%	0 a
<i>Moringa</i> 15 + K2%	0 a
<i>Moringa</i> 15 + K3%	0 a
<i>Moringa</i> 20 + K1%	0 a
<i>Moringa</i> 20 + K2%	0 a
<i>Moringa</i> 20 + K3%	0 a
<i>Moringa</i> 5	54.1 d
<i>Moringa</i> 10	37.2 c
<i>Moringa</i> 15	20.6 b
<i>Moringa</i> 20	13.7 b
K1% alone	75.3 e
K2% alone	69.0 e
K3% alone	70.4 e
unamended PDA (control)	88.1 f

<sup>1</sup>*Moringa oleifera* extract at different concentrations (5, 10, 15 and 20 g/10 ml solution) was combined with different doses of kerosene, respectively and incorporated in PDA. K1%, 2% and 3% means 0.1; 0.2 and 0.3 ml of kerosene was added to 10 ml water, respectively, to prepare the extract. <sup>2</sup>Each value is a mean of five replicates. Values not followed by the same letters are significantly different ( $P = 0.05$ ) according to Student Newman Keuls.

**Table 8.2**

Effect of seed treatments with *Moringa oleifera* leaf extracts on the severity, incidence and control of the *Sclerotium rolfsii* damping-off and stem rot of cowpea in the greenhouse

Treatments <sup>1</sup>	Severity <sup>2,5</sup>		Disease incidence <sup>3,5</sup> (%)		Disease control <sup>4,5</sup> (%)	
	Tchawé kpayo	IT89KD 374-57	Tchawé kpayo	IT89KD 374-57	Tchawé kpayo	IT89KD 374-57
<i>Moringa</i> 5 + KS <sup>1</sup>	3 bcde	4.3 hi	35.9 f	32.9 de	64.1 e	66.7 ef
<i>Moringa</i> 10 + KS	1.2 ab	0.9 b	11.0 b	9.0 b	89.0 i	90.9 h
<i>Moringa</i> 15 + KS	0.0 a	0.1 a	0.0 a	0.0 a	100.0 j	100 i
<i>Moringa</i> 20 + KS	0.0 a	0.0 a	1.1 a	0.0 a	98.9 j	100 i
<i>Moringa</i> 5 + K	3.9 cdef	4.1 ghi	45.9 h	50.9 f	54.1 c	48.5 d
<i>Moringa</i> 10 K	3.1 bcde	3.7 fg	32.8 ef	30.0 d	67.2 ef	69.7 f
<i>Moringa</i> 15 + K	2.6 bcde	3.1 de	24.9 de	19.0 c	75.1 fg	80.8 g
<i>Moringa</i> 20 + K	1.9 abc	2.9 d	16.1 bc	17.9 c	83.9 hi	81.8 g
<i>Moringa</i> 5 + S	4.4 ef	3.1 de	48.2 h	51.9 f	51.8 c	47.5 d
<i>Moringa</i> 10 + S	3.2 bcde	4.5 ij	30.9 ef	31.9 de	69.1 ef	67.7 ef
<i>Moringa</i> 15 + S	2.0 abc	2.9 c	22.0 cd	16.1 c	78.0 gh	83.8 g
<i>Moringa</i> 20 + S	2.2 bcd	2.3 c	19.0 cd	20.0 c	81.0 gh	79.8 g
<i>Moringa</i> 5	4.4 ef	4.6 ij	60.0 i	57.9 g	40.0 b	41.4 c
<i>Moringa</i> 10	4.3 def	3.5 ef	44.9 gh	46.9 f	55.1 cd	52.5 d
<i>Moringa</i> 15	3.2 bcde	3.5 ef	37.2 fg	36.0 e	62.8 de	63.6 e
<i>Moringa</i> 20	2.6 bcde	3.8 fgh	33.9 f	30.0 d	66.1 e	69.7 f
K	4.3 def	4.2 ghi	94.9 j	97.8 i	5.1 a	1 a
S	5.5 f	4.1 ghi	97.0 j	100.0 i	3.0 a	0 a
Water	4.5 ef	4.6 ij	100.0 j	99.1 i	0.0 a	0 a
Super-Homai	3.3 bcde	3.5 ef	66.0 i	72.8 h	34.8 b	26.3 b
Untreated control	5.7 f	5.0 j	100.0 j	98.9 i	0.0 a	0 a

<sup>1</sup>*Moringa*5, 10, 15 or 20+KS refers to the weight of *M. oleifera* leaves (5, 10, 15 or 20 kg, respectively) that was crushed, suspended in 10 l sterile water, mixed with K (kerosene 1 %) and S (Marseilles soap 0.1 % w/v), stored overnight and filtered through a sterile cloth to get the final extract. K = kerosene only; S = Soap only. Super-Homai is a registered fungicide in Benin and was used as positive control. <sup>2</sup>Severity was rated on a scale of 0 to 6 (Adandonon, 2000). Each value is a mean of four replicates. <sup>3</sup>Disease incidence expressed as percentage diseased plants. <sup>4</sup>Percentage disease control by each treatment is calculated as follows: DR % = [1 - (DT/DC)] \* 100 where DR = Disease reduction or control; DC = Disease incidence on the control unit (untreated) and DT = Disease incidence on the treatment unit. <sup>5</sup>In the same column, means followed by the same letter are not significantly different ( $P = 0.05$ ) according to the General Linear Model (GLM) procedure and mean separations were done using the Student Newman Keuls option.



**Table 8.3**

Effect of seed treatments with *Moringa oleifera* leaf extracts on the incidence and control of the *Sclerotium rolfsii* damping-off and stem rot of cowpea in the field

Treatments <sup>1</sup>	Disease incidence <sup>2,4</sup> (%)		Disease control <sup>3,4</sup> (%)	
	Tchawé kpayo	IT89KD 374-57	Tchawé kpayo	IT89KD 374-57
<i>Moringa</i> 5 + KS	12.2 fg	13.4 fgh	13.5 bc	19.3 efg
<i>Moringa</i> 10 + KS	8.8 bc	9.7 d	37.6 fg	41.6 i
<i>Moringa</i> 15 + KS	4.4 a	5.2 a	68.8 h	68.7 l
<i>Moringa</i> 20 + KS	5.0 a	4.9 a	64.5 h	70.5 l
<i>Moringa</i> 5 + K	14.8 h	14.2 hi	0 a	14.5 de
<i>Moringa</i> 10 + K	12.4 g	13.9 ghi	12.1 b	16.3 def
<i>Moringa</i> 15 + K	10.2 de	9.5 cd	27.7 de	42.8 ij
<i>Moringa</i> 20 + K	8.2 b	7.7 b	41.8 g	53.6 k
<i>Moringa</i> 5 + S	14.3 h	15.3 jk	0 a	7.8 bc
<i>Moringa</i> 10 + S	13.1 gh	12.4 ef	7.1 ab	25.3 gh
<i>Moringa</i> 15 + S	9.5 cd	10.1 d	32.6 ef	39.2 i
<i>Moringa</i> 20 + S	9.3 bcd	8.5 bc	34 efg	48.8 jk
<i>Moringa</i> 5	14.5 h	14.0 hi	0 a	15.7 de
<i>Moringa</i> 10	14.1 h	12.9 efg	0 a	22.3 fgh
<i>Moringa</i> 15	10.0 d	12.1 e	29.1 e	27.1 h
<i>Moringa</i> 20	11.2 ef	10.0 d	20.6 cd	39.8 i
Kerosene	14.9 h	15.2 jk	0 a	8.4 bc
Soap	15.0 h	16.6 l	0 a	0 a
Water	15.2 h	15.8 kl	0 a	4.8 ab
Super-Homai	12.3 fg	14.7 ij	12.8 bc	11.4 cd
Untreated control	14.1 h	16.6 l	0 a	0 a

<sup>1</sup>*Moringa*5, 10, 15 or 20+KS refers to the weight of *M. oleifera* leaves (5, 10, 15 or 20 kg, respectively) that was crushed, suspended in 10 l sterile water, mixed with K (kerosene 1 %) and S (Marseilles soap 0.1 % w/v), stored overnight and filtered through a sterile cloth to get the final extract. K = kerosene only; S = Soap only. Super-Homai is a registered fungicide in Benin and was used as positive control. <sup>2</sup>Disease incidence expressed as percentage diseased plants. <sup>3</sup>Percentage disease control by each treatment is calculated as follows: DR % = [1 - (DT/DC)] \* 100 where DR = Disease reduction or control; DC = Disease incidence on the control unit (untreated) and DT = Disease incidence on the treatment unit. Each value is a mean of four replicates. <sup>4</sup>In the same column, means followed by the same letters are not significantly different ( $P = 0.05$ ) according to the General Linear Model (GLM) procedure and mean separations were done using the Student Newman Keuls option.

## **CHAPTER 9**

# **BIOCONTROL AGENTS IN COMBINATION WITH *MORINGA* *OLEIFERA* EXTRACT FOR INTEGRATED CONTROL OF *SCLEROTIUM*-CAUSED COWPEA DAMPING-OFF AND STEM ROT IN THE FIELD**

**BIOCONTROL AGENTS IN COMBINATION WITH *MORINGA OLEIFERA*  
EXTRACT FOR INTEGRATED CONTROL OF *SCLEROTIUM*-CAUSED  
COWPEA DAMPING-OFF AND STEM ROT**

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**Abstract**

Damping-off and stem rot caused by *Sclerotium rolfsii* are harmful to cultivated plants in moist tropics and in warm temperate areas. Greenhouse and field trials were conducted to evaluate the effectiveness of seed treatments, soil drench and soil sprinkle of *Trichoderma* Kd 63 and *Bacillus subtilis*, soil sprinkle of *Trichoderma* IITA 508 and seed treatments of *Moringa oleifera* L. for the control of cowpea damping-off and stem rot caused by *S. rolfsii*. Percentage diseased plants, and disease severity were recorded. In the greenhouse, results showed that seed treatments with *M. oleifera* leaf extracts and *Trichoderma* Kd 63, and soil sprinkle of *Trichoderma* IITA 508 have significant ( $P = 0.05$ ) effect on the disease incidence, compared to other treatments or controls. The disease severity followed the same pattern. However, given a type of treatment, effects of *Trichoderma* applications were significantly higher than those of *Bacillus*. Moreover, considering a biological control agent, seed treatments yielded the lowest percentage diseased plants. A remarkable improvement in the performance of the antagonists was observed when *M. oleifera* seed treatments were combined with *Trichoderma* or *Bacillus* soil sprinkle. A similar trend was observed in the two-year experiment in the field. *Moringa oleifera* seed treatment combined with *Trichoderma* soil sprinkle significantly decreased the disease incidence and increased the disease control and yield in the field, although less efficiently than under greenhouse conditions. This is the first report of *M. oleifera* seed treatments combined with *Trichoderma* soil sprinkle as an integrated control of damping-off and stem rot of cowpea in the field.

Keywords: *Bacillus subtilis*, integrated control, *Moringa oleifera*, *Sclerotium rolfsii*, *Trichoderma harzianum*, *Vigna unguiculata*.

## 9.1 Introduction

*Sclerotium rolfsii* Sacc. (teleomorph: *Athelia rolfsii* (Curzi) Tu & Kimbrough) is a soilborne plant pathogenic fungus that causes diseases in over 500 plant species throughout the world (Punja, 1985; Harlton *et al.*, 1995; Cilliers *et al.*, 2000). Recently, *S. rolfsii* was also reported as the causal agent of damping-off and stem rot on cowpea, *Vigna unguiculata* (L.) Walp, in the Ouémé Valley, Benin (Adandonon, 2000; Kossou *et al.*, 2001) where yield losses reached up to 40 % (PEDUNE-BENIN, 1995). Control of *Sclerotium* diseases may be achieved by cultural means (Singh and Allen, 1979; Singh *et al.*, 1997) or by using chemical fertilisers or by applying fungicides to the soil before planting or in the furrow during planting (Punja, 1985), or as a soil drench or in-furrow spray near the roots of crops (Jones, 1987). In Benin, the only registered fungicide used on edible crops such as cowpea is Super-Homai 70 % PM (active ingredient: methylthiophanate 35 %, thiram 20 %, and diazinon 15 %) (SPV, Benin). Unfortunately, there is still a problem regarding its efficacy (Kakpo-Zannou, B. L., Pers. comm.). Despite the efficacy of synthetic fungicides, the widespread use of chemicals has become a subject of public concern, mainly due to their potential harmful effect on non-target organisms, the development of resistant races of pathogens, and the possible carcinogenicity of some chemicals (Zaki *et al.*, 1998). Thus, there is a need to examine the potential for non-synthetic chemical approaches to disease management.

Research has demonstrated that plant extract-based (Stoll, 1988; Singh *et al.* 1980) or biological control, through the use of beneficial fungi or bacteria (Cook and Baker, 1983; Datnoff *et al.*, 1995) are potentially feasible alternatives to the use of fumigants or fungicides. Leaf extracts from plants such as *M. oleifera* have been reported to have some fungicide effects against *S. rolfsii* diseases in many crops in the greenhouse. Moreover, floras with demonstrated antagonistic properties under field conditions include fungi, principally *Trichoderma* spp. and bacteria such as *Bacillus* spp. (Campbell, 1989; Datnoff *et al.*, 1995; Leifert *et al.*, 1996; Li *et al.*, 1998). Almeida and Landim (1981) reported that *Trichoderma* spp. reduced *S. rolfsii* disease incidence in the greenhouse whereas Hadar *et al.* (1979; 1981) indicated that *Trichoderma harzianum* Rifai was used as a biocontrol agent against *R. solani* and *S. rolfsii* under both greenhouse and field conditions. *Bacillus* spp. have long been evaluated for control of diseases, including root diseases (Capper and Campbell, 1986) and postharvest diseases (Singh and Daverall, 1984; Huang *et al.*, 1992). However, quite often, the biological control is less effective than many of the commercial fungicides currently in

use (Pusey, 1994). The often unpredictable or variable activity of the biocontrol agent compared with the fungicide is one of the reasons why biocontrol has not gained a large market share in comparison to fungicides in the field (Li *et al.*, 1998).

Although several species of *Trichoderma* have been evaluated in the control of *S. rolfsii* in various crops (Papavizas, 1985), *Bacillus subtilis* (Ehrenberg) Cohen of many root pathogens (Massomo *et al.*, 2004) and *M. oleifera* of some soilborne diseases (Stoll, 1988; SIBAT, 1993), there are no reports of these biocontrol agents or *M. oleifera* being utilised to protect cowpea seedlings against damping-off and stem rot caused by *S. rolfsii* in the field. Moreover, no work has been reported to determine the compatibility of *Trichoderma* or *Bacillus* with *M. oleifera* extracts, and the possible improvement in biocontrol activity that might result from such integration.

The main objective of this work was to develop appropriate formulations of *T. harzianum*, *B. subtilis* and *M. oleifera* extracts as a plant-based product, and to evaluate their performance either on their own, or in combination with *M. oleifera* for control of *Sclerotium*-caused damping-off and stem rot of cowpea in the greenhouse and field.

## 9.2 Material and methods

### 9.2.1 Pathogen culture and inoculum

*Sclerotium rolfsii* isolate IITA 409 was isolated from diseased cowpea plants collected in 2001 from the Ouémé valley. *S. rolfsii* was sub-cultured and maintained on potato dextrose agar (PDA) slants at 4°C.

The pathogen inoculum was prepared using the millet seed inoculum technique (Weideman and Wehner, 1993), described in Chapter 3.

### 9.2.2 Biological control agents (BCA) and formulation

*Trichoderma* IITA 508 (isolated from a diseased cowpea stem, Cotonou, Benin), *Trichoderma* Kd 63 (kindly donated by Dr M. Morris, Plant Health Products, Pietermaritzburg, South Africa) and *B. subtilis* (kindly donated by Prof P. L. Steyn, Stimuplant CC, Mooiplaats, Pretoria, South Africa) were used. *Trichoderma* IITA 508 had shown *in vitro* inhibitory action against *S. rolfsii* in a previous study (Adandonon, 2000). *Trichoderma* Kd 63 and *B. subtilis* were in powder formulation with 10<sup>9</sup> colony forming units (cfu)/g powder, respectively. Cell suspensions were prepared using five grams of *Trichoderma* Kd 63 or *B. subtilis* powder per litre distilled water, as recommended by the manufacturer.

### 9.2.3 Plant-based product

Fresh *M. oleifera* leaves were collected and extracts were made according to the procedure described in Chapter 8.

### 9.2.4 Cowpea plant material

A susceptible local cowpea cultivar, Tchawé kpayo, obtained from the Ouémé valley, Benin was used. Before planting, the seeds were surface sterilized in 1 % NaOCl for 2 min and rinsed in sterile distilled water.

### 9.2.5 Effect of powder dosage of mixed *Trichoderma* Kd 63 and *B. subtilis* soil treatment on damping-off and stem rot in the greenhouse

The soil inoculation was done 2 d before planting by mixing 10 g of the *S. rolfisii* millet seed inoculum with 1 kg steam-disinfected sandy loam soil in a pot.

Three dosages (0.05, 0.1 and 0.15 g powder) of each BCA (*Trichoderma* Kd 63 and *B. subtilis*) were tested. Each dosage was mixed with 5 g disinfected soil and sprinkled into the planting furrow at planting. There were three controls and four replicates: control 1 was pasteurised soil mixed with 10 g inoculated steeped millet seeds, control 2 was pasteurised soil without steeped millet seed and control 3 was seed treatment with the fungicide Super-Homai.

### 9.2.6 Comparative effects of seed treatment, soil drench and sprinkle of biocontrol agents alone or combined with *Moringa oleifera* extracts seed treatment on damping-off and stem rot in the greenhouse

Disinfected soil was treated with *S. rolfisii* millet seed-based inoculum 2 d before planting, as described earlier. Treatments included: seed treatment, soil drench, and soil sprinkle of *Trichoderma* Kd 63 and *B. subtilis*, respectively; soil sprinkle of *Trichoderma* IITA 508 and seed treatment of *M. oleifera* extracts. In the integrated control evaluation, seeds were first treated with *M. oleifera* leaf extract solutions and planted before the soil mixed with BCA was sprinkled into the planting furrow.

A suspension of *Trichoderma* Kd 63 and *B. subtilis* were soil-drenched at 3 ml solution per planting furrow shortly after cowpea seed planting. Before planting, seed treatments consisted of soaking cowpea seeds in *Trichoderma* Kd 63 or *B. subtilis* cell suspension (5 g/l) and in *M. oleifera* leaf extract solutions for 5 min, respectively. The seeds were left to dry and then planted.

In the powder dosage experiment, there was no difference ( $P = 0.05$ ) among all three tested dosages of *Trichoderma* whereas a *Bacillus* dosage of 0.1 or 0.15 g powder per 5 g soil yielded less diseased plants than that of 0.05 g per 5 g soil. For further experiments, the *Trichoderma* Kd 63 and *Bacillus* powder dosage of 0.05 g and 0.1 g, respectively, per 5 g soil per furrow were evaluated. A millet seed-based formulation of *Trichoderma* IITA 508 was done as described earlier with *S. rolfsii*. The *T. harzianum* IITA 508 millet seed inoculum was sprinkled at the rate of 0.1 g per 5 g soil per furrow. This inoculum dosage of *T. harzianum* IITA 508 was based on a preliminary study (Adandonon, unpublished).

### 9.2.7 Experimental design and planting

The experimental design was a randomised block design with 11 treatments, three controls and four replicates each. Control 1 was pasteurised soil mixed with 10 g inoculated steeped millet seeds and control 2 was pasteurised soil without steeped millet seeds whereas control 3 was treatment with Super-Homai 70 % PM. Seeds were respectively treated as mentioned earlier (9.2.6) and planted in the greenhouse at four seeds per pot (14 cm diam. x 18 cm height). Pots were incubated at temperatures varying between 26-30 °C and relative humidity between 65-90 %. Pots were watered every second day.

### 9.2.8 Data collection and Koch's Postulates

The number of damping-off seedlings and stem rot plants was visually recorded 2 d after planting and every day thereafter until 30 d after planting. The percentage disease control per treatment is calculated as follows:

$$DR \% = [1 - (DT/DC)] * 100$$

where DR : Disease control or reduction; DT : Disease incidence on the treatment unit  
DC : Disease incidence on the control unit (zero treatment).

The symptoms on plants were rated using a scale of 0 to 6 to determine the disease severity (Adandonon, 2000) as follows: 0-: No visible symptoms; 1-: Leaves did not wilt; plants fell over on the ground after the 4<sup>th</sup> d; 2-: Leaves wilted on 3<sup>rd</sup> d; plants fell over on the ground on 4<sup>th</sup> d; 3-: Leaves wilted on 3<sup>rd</sup> d; plants fell over on the ground on 3<sup>rd</sup> d; 4-: Leaves wilted on 2<sup>nd</sup> d; plants fell over on the ground on 2<sup>nd</sup> or 3<sup>rd</sup> d; 5-: Leaves wilted on 1<sup>st</sup> d; plants fall over on the ground on 2<sup>nd</sup> d and; 6-: Leaves wilted; plants fell over on the ground within 24 h.

To fulfil Koch's postulates, dying seedlings were removed at each observation, and at least one plant from each pot was assayed to verify the presence of the appropriate fungal species (Brantner and Windels, 1998). The reisolated fungus was cultured on PDA and colony characteristics were recorded and compared to the original isolates.

#### 9.2.9 Statistical analysis

The statistical analysis was performed as described in Chapter 3.

#### 9.2.10 Field experiment

The experiment was conducted in the field in 2001 and 2002 to test the performance of the treatments and correlate greenhouse and field experiment results. The treatments included all 11 treatments mentioned earlier (9.2.6) and two controls. Control 1 was a plot planted with untreated seeds whereas control 2 was seeds treated with Super-Homai.

#### *Experimental design, planting, data collection and statistical analysis*

The experimental design was a randomised block design with two controls and four replicates. Two cowpea cultivars, namely Tchawé kpayo and IT89KD-374-57, were used. Each treatment was assigned to a plot of 200 m<sup>2</sup>. The plots were planted as mentioned earlier (9.2.7) and weeded by the farmers themselves using traditional cultural practices. The plant intervals were those of farmers. Data collection and analysis were done as described earlier (Chapter 3).

### **9.3 Results**

#### 9.3.1 Effect of powder dosage of mixed *Trichoderma* Kd 63 and *B. subtilis* soil treatment on damping-off and stem rot in the greenhouse.

Percentage diseased plants recorded were significantly less ( $P = 0.05$ ) in soil treated with *Trichoderma* Kd 63, compared to other treatments (Table 9.1). There was no significant difference ( $P = 0.05$ ) among all powder dosages of *Trichoderma* Kd 63 tested. *Bacillus* powder dosage of 0.1 or 0.15 per 5 g soil per furrow yielded significantly ( $P < 0.05$ ) less diseased plants than that of 0.05 g soil per furrow. No significant difference ( $P = 0.05$ ) was detected between the *B. subtilis* dosage of 0.05 g per 5 g soil per furrow, Super-Homai and the untreated, infested control. Disease severity followed a similar pattern (Table 9.1).



### 9.3.2 Comparative effects of seed treatment, soil drench and sprinkle of biocontrol

agents alone or combined with *Moringa oleifera* extracts seed treatment on damping-off and stem rot in the greenhouse

Given a type of treatment (soil drench, seed treatment or soil sprinkling), *Trichoderma* treatment effects were significantly higher than those of *Bacillus*. Moreover, considering a biological control agent (*Trichoderma* Kd 63 or *B. subtilis*), seed treatments yielded the lowest percentage diseased plants, followed by the soil sprinkling and soil drench treatments. Results presented in Table 9.2 show that seed treatments of *M. oleifera* leaf extracts, seed treatments of *Trichoderma* Kd 63 and soil sprinkle of *Trichoderma* IITA 508 had significant ( $P = 0.05$ ) effects on the disease incidence, when compared to other treatments, untreated *Sclerotium*-inoculated pasteurised soil (control) and Super-Homai (fungicide) treatments. A remarkable improvement in the performance of the antagonists was observed when *M. oleifera* seed treatments were combined with *Trichoderma* or *Bacillus* soil sprinkles (Table 9.2).

### 9.3.3 Field experiment

The air temperature and relative humidity recorded in the field between 08h00 and 14h00 varied between 25.7 to 27.8 °C and 62 to 97 %, respectively in the 2002 field experiment. The disease incidence recorded with the untreated control was the highest (16.8 %) compared to all treatments (Table 9.3). The trend of the disease incidence for the treatments in the field was similar to that recorded in the greenhouse. Seed treatment with *M. oleifera* leaf extracts and *Trichoderma* Kd 63, and soil sprinkle of *Trichoderma* IITA 508 millet seed inoculum yielded the lowest percentage of diseased plants and the highest percentage disease control, regardless of the cultivars (Table 9.3). The disease control trend of the treatments in the field was similar to but less than that in the greenhouse (Table 9.2 and 9.3). At harvest, all biological control agents or *M. oleifera* extracts, when applied alone, performed significantly better than the untreated control in terms of recorded yields in the field. However, the highest yields were recorded when *M. oleifera* extract seed treatment was combined with soil sprinkles of *Trichoderma* Kd 63 or *Trichoderma* IITA 508 or *B. subtilis*. The trend was similar for both cultivars (Table 9.3).

Disease incidence, control and yield, and variation of the recorded climate parameters were less in the second year (2002) experiment in the field.

#### 9.4 Discussion

The use of plant extracts and biocontrol agents is seen as a viable method for controlling plant diseases (Ark and Thompson, 1959; Leifert *et al.*, 1996; Kilian *et al.*, 2000). The results obtained in the greenhouse, in the powder dose experiment showed that *Trichoderma* Kd 63 and *Bacillus subtilis* had a significant effect on damping-off and stem rot incidence at 0.05 g and 0.1 g inoculum per 5 g of soil per furrow, respectively. When applied at low dose into the soil, the further growth of the biocontrol agent (*B. subtilis*) might not be high enough to control the pathogen population whereas a significant ( $P < 0.05$ ) effect was observed at a higher dose. Early reports indicated that the biocontrol efficacy depends on the agent used, and its effect positively correlates with its concentration and negatively with the pathogen population (Elad *et al.*, 1980a). *Trichoderma harzianum* Kd 63 seed treatment, *Trichoderma* IITA 508 millet seed inoculum soil sprinkle and *M. oleifera* leaf extract seed treatment yielded significant disease control and were more effective than *Bacillus* treatments in the greenhouse. The effects of *Trichoderma* on many pathogens are well documented (Papavizas and Lumsden, 1980; Cook and Baker, 1983; Weidman and Wehner, 1993). The effectiveness of *Trichoderma* lies in a combination of competition, antifungal metabolites, toxic antibiotic and mycoparasitism (Mukherjee and Raghu, 1997; Howell, 2003). This might explain the efficacy of *Trichoderma* observed in the present study. *Bacillus subtilis* is often used as seed treatments in the control of root diseases (Capper and Campbell, 1986). However, early reports indicated that *B. subtilis* is often very variable with very different results in different locations, or even different parts of a season in the same location (Campbell, 1989). This might explain the low efficacy of *B. subtilis* observed in the current work. *Moringa oleifera* leaf extracts were reported to inhibit the *in vitro* growth of *Pythium* sp., causal agent of damping-off of legumes and vegetables (SIBAT, 1993) and reduce soilborne disease incidence in cereals (Stoll, 1988). *Moringa oleifera* leaves are said to contain some crystalline alkaloids (SIBAT, 1993), fatty acid, proteins, glycosides and niazirin, said to be responsible for antimicrobial activities (<http://www.essentialdrugs.org/emed/archive/200303/msg00042.php>). This might explain the results obtained in the present study.

Given a biological agent species, seed treatments performed better than food-based powder or milled seed inoculum mixed with soil sprinkle, which, in turn, performed better than the soil drench treatments. This result shows that the type of biological

formulation affects the efficacy of the agent, as reported earlier (Hadar *et al.*, 1979; Sivan *et al.*, 1984). *Trichoderma*, used as a seed treatment, was reported to protect many vegetable crops from damping-off diseases induced by *Pythium* sp., *Rhizoctonia solani* and *S. rolfsii* (Chet and Baker, 1980; Adams, 1990; Hornby, 1990).

*Moringa oleifera* combined with *Trichoderma* (and to some extent with *Bacillus*) resulted in the best disease control in the field, although less effective than under greenhouse conditions. When seeds were treated with *M. oleifera*, the fungicidal active ingredients in the extracts might be toxic to the pathogen, act systemically, and therefore protect the seedlings against attack from the pathogen. This action might have been reinforced when *M. oleifera* was combined with a soil treatment with *Trichoderma*. As a result, the synergistical effect of *Trichoderma* and *M. oleifera* protected the further growth of the plant against infection by the pathogen. Although *T. harzianum* combined with synthetic chemicals was reported earlier (Elad *et al.*, 1980b), to our knowledge, no research has been done using plant extracts, especially *M. oleifera* leaf extracts combined with *Trichoderma* for disease control in the field. The results here are important since *M. oleifera* leaves are found all over Benin and the extracts are easy to prepare. Moreover, *Trichoderma* IITA 508 was collected from *Sclerotium*-infected field in the valley, so it is in its native ecosystem. Farmers could use the combination to reduce the yield losses inflicted by the diseases to the crop, and therefore increase their income.

This is the first report of *M. oleifera* seed treatments combined with *Trichoderma* soil treatments for integrated control of damping-off and stem rot of cowpea in the field. The present results are of interest since they point to the high possibility of plant extract-based and biological control of *S. rolfsii* in the field. This could be an alternative to the use of synthetic fungicide treatments (Harman *et al.*, 1980; 1981). Further work will focus in increasing the efficacy of the extracts in the field and also determining the biologically active ingredient of the *M. oleifera* extracts and mode of action.

## 9.5 Acknowledgements

Research was financed by the International Institute of Tropical Agriculture (IITA), Nigeria. Thanks are due to Dr M. Morris, Plant Health Products, Pietermaritzburg and Prof P. L. Steyn, Stimuplant CC, Mooiplaats, Pretoria, South Africa for providing with *Trichoderma* Kd 63 and *Bacillus subtilis*, respectively.

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**Table 9.1**

Effect of different dosages of *Trichoderma* Kd 63 and of *B. subtilis* on the damping-off and stem rot incidence and severity of cowpea inoculated with *Sclerotium rolfsii* in the greenhouse

Treatments <sup>1</sup>	Diseased plants (%)	Severity <sup>2</sup>
<i>Trichoderma</i> Kd 63, (0.05)	20.8 b	1.3 b <sup>3</sup>
<i>Trichoderma</i> Kd 63, (0.10)	16.7 b	1.1 b
<i>Trichoderma</i> Kd 63, (0.15)	21.0 b	1.0 b
<i>Bacillus</i> (0.05)	83.3 de	3.8 d
<i>Bacillus</i> (0.10)	56.7 c	2.1 c
<i>Bacillus</i> (0.15)	59.2 c	1.9 c
Control 1	95.8 e	4.9 e
Control 2	0 a	0 a
Control 3	80.4 d	3.4 d

<sup>1</sup>Powder inoculum of *Trichoderma* Kd 63 and *B. subtilis* were respectively sprinkled into the planting furrow at a rate of 0.05, 0.1 and 0.15 g inoculum per 5 g soil per furrow. Control1 = pasteurised soil mixed with 10 g *Sclerotium*-inoculated steeped millet seeds (nontreated, infested control); control2 = uninoculated pasteurised soil and Control3 = Super Homai 70 % PM (methylthiophanate 35 %, thiram 20 %, and diazinon 15 %). <sup>2</sup>Severity was recorded as symptoms on plants rated on a scale of 0 to 6 (Adandonon, 2000). <sup>3</sup>In the same column, means followed by the same letter are not significantly different ( $P = 0.05$ ) according to the General Linear Model (GLM) procedure and mean separations were done using Student Newman Keuls option.



**Table 9.2**

Effect of *Trichoderma* Kd 63, *Trichoderma* IITA 508, *Bacillus subtilis* and *Moringa oleifera* leaf extracts on the incidence, severity and control of *Sclerotium* damping-off and stem rot of cowpea in the greenhouse

Treatments <sup>1</sup>	Diseased plants <sup>2,5</sup> (%)	Severity <sup>3,5</sup>	Disease control <sup>4,5</sup> (%)
<i>Trichoderma</i> Kd 63 soil drench	38.9 f	2.7 g	60.14 de
<i>Bacillus</i> soil drench	49.8 g	3.9 h	48.97 cd
<i>M. oleifera</i> leaf extract seed treatment ( <i>Moringa</i> ST)	5.5 abc	1.1 cde	94.36 hij
<i>Trichoderma</i> Kd 63 seed treatment	13.9 bc	1.3 de	85.76 ghi
<i>Bacillus</i> seed treatment	32.7 de	2.7 g	66.50 ef
<i>Trichoderma</i> IITA 508 soil sprinkling (TiSS)	15.3 cd	1.5 ef	84.32 gh
<i>Trichoderma</i> Kd 63 soil sprinkling (TkSS)	25.1 de	1.9 f	74.28 fg
<i>Bacillus</i> soil sprinkling (BSS)	56.8 g	3.2 g	41.80 c
<i>Moringa</i> ST + TiSS	1.2 a	0.5 ab	98.77 j
<i>Moringa</i> ST + TkSS	2.5 a	0.7 bc	97.44 ij
<i>Moringa</i> ST + BSS	4.3 ab	0.8 bcd	95.59 hij
<i>Sclerotium</i> -inoculated pasteurised soil	97.6 i	5.4 i	0 a
Uninoculated pasteurised soil	0 a	0 a	100 j
Super Homai.	79.7 h	3.8 h	18.34 b

<sup>1</sup>*Trichoderma* Kd 63 and *B. subtilis* soil drench was done at 3 ml solution per planting furrow shortly after cowpea seed planting; *Moringa* ST = *M. oleifera* leaf extract seed treatment; *Trichoderma* Kd 63, *Bacillus* and *M. oleifera* seed treatments consisted in soaking for 5 min cowpea seeds in *Trichoderma* Kd 63, *Bacillus* cell suspension (5 g/l) and in *M. oleifera* extract solution, respectively; TiSS, TkSS and BSS = *Trichoderma* IITA 508 millet seed inoculum, *Trichoderma* Kd 63 powder inoculum and *B. subtilis* powder inoculum mixed with soil sprinkling at 0.1, 0.05 and 0.1 g inoculum per 5 g soil into each planting furrow. <sup>2</sup>Diseased plants (%) = number of diseased plant from a specific unit out of the total number of plants from the unit multiplied by 100. <sup>3</sup>Severity was recorded as symptoms on plants rated on a scale of 0 to 6 as follows (Adandonon, 2000). <sup>4</sup>Percentage disease control by each treatment is calculated as follows: DR % = [1 - (DT/DC)] \* 100 where DR = Disease reduction or control; DC = Disease incidence on the control unit (zero treatment) and DT = Disease incidence on the treatment unit. <sup>5</sup>In the same column, means follow by the same letters are not significantly different ( $P = 0.05$ ) according to the General Linear Model (GLM) procedure and mean separations were done using SNK option.

**Table 9.3**

Effects of *Moringa oreifera* leaf extracts, *Trichoderma harzianum* Kd 63, IITA 508 and *Bacillus subtilis* as plant-based products and biological control agents on *Sclerotium* damping-off and stem rot of cowpea, in terms of incidence, control and grain yield in the field in 2002

Treatments <sup>1</sup>	Disease incidence <sup>2,4</sup> (%)		Disease control <sup>3,4</sup> (%)		Grain yield <sup>4</sup> (Kg/ha)	
	Tchawé kpayo	IT89KD- 374-57	Tchawé kpayo	IT89KD- 374-57	Tchawé kpayo	IT89KD- 374-57
<i>Trichoderma</i> Kd 63 soil drench	12.2 ef	13.9 f	29.48 de	17.26 b	679.2 <sup>2</sup> bc <sup>3</sup>	658.1 c
<i>Bacillus</i> soil drench	14.4 gh	15.8 fg	16.76 bc	5.95 ab	641.1 ab	650.0 c
<i>Moringa</i> ST	8.3 cd	6.1 bc	52.02 fg	63.69 ef	791.4 de	776.4 f
<i>Trichoderma</i> Kd 63 seed treatment	9.1 d	9.8 de	47.40 f	41.67 cd	775.2 d	764.2 f
<i>Bacillus</i> seed treatment	12.8 efg	11.7 e	26.01 cde	30.36 c	683.5 c	690.1 d
<i>Trichoderma</i> IITA 508 SS (TiSS)	6.9 bc	7.8 cd	60.12 gh	53.57 de	804.7 de	810.3 g
<i>Trichoderma</i> Kd 63 SS (TkSS)	11.3 e	10.9 e	34.68 e	35.12 c	698.1 c	724.5 e
<i>Bacillus</i> soil sprinkling (BSS)	13.5 fg	14.7 fg	21.97 cd	12.50 ab	665.2 bc	644.2 bc
<i>Moringa</i> ST + TiSS	2.5 a	1.8 a	85.55 i	89.29 g	965.3 g	987.3 i
<i>Moringa</i> ST + TkSS	5.1 b	4.9 b	70.52 h	70.83 f	858.4 fg	835.4 h
<i>Moringa</i> ST + BSS	7.4 cd	6.5 bc	57.22 fg	61.31 ef	821.6 ef	843.6 h
Zero treatment (Control)	17.3 i	16.8 g	0.00 a	0.00 a	625.8 a	590.5 c
Super-Homai	15.9 hi	14.5 fg	8.09 ab	13.69 b	640.5 ab	627.4 b

<sup>1</sup>*Trichoderma* Kd 63 and *B. subtilis* soil drench were done at 3 ml solution per planting furrow shortly after cowpea seed planting; *Moringa* ST = *M. oreifera* leaf extract seed treatment; *Trichoderma* Kd 63, *Bacillus* and *M. oreifera* seed treatments consisted in soaking for 5 min cowpea seeds in *Trichoderma* Kd 63, *Bacillus* cell suspension (5 g/l) and in *M. oreifera* extract solution, respectively; TiSS, TkSS and BSS = *Trichoderma* IITA 508 millet seed inoculum, *Trichoderma* Kd 63 powder inoculum and *B. subtilis* powder inoculum mixed with soil sprinkling at 0.1, 0.05 and 0.1 g inoculum per 5 g soil into each planting furrow. “+” = combination of treatments. Super –Homai.

<sup>2</sup>Diseased incidence (%) = number of diseased plant from a specific unit out of the total number of plants from the unit multiplied by 100.

<sup>3</sup>Percentage disease control by each treatment is calculated as follows: DR % = [1 - (DT/DC)] \* 100 where DR = Disease reduction or control; DC = Disease incidence on the control unit (zero treatment) and DT = Disease incidence on the treatment unit. Each value is a mean of four replicates. In the same column, means follow by the same letters are not significantly different (P = 0.05) according to the General Linear Model (GLM) procedure and mean separations were done using SNK option.

## **CHAPTER 10**

# **GENERAL DISCUSSION**

GENERAL DISCUSSION

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Cowpea, *Vigna unguiculata* (L.) Walp, is a major crop in many tropical countries (Fernando and Linderman, 1993) and in Benin cowpea is cultivated throughout the country. It constitutes a cash crop for farmers in the Ouémé valley where it is produced after natural flooding. The cowpea yield recorded in the farmers' field is often low due to a range of pests and diseases including damping-off and stem rot. The diseases inflict up to 40 % yield losses to the crop in the valley. During a farmer's day trip in the north of Benin, symptoms of the disease were observed but cowpea framers did not differentiate the symptoms from injury due to weeding. This misunderstanding of the disease by the farmers emphasises the threat the diseases represent for the cowpea production in the future. The main objectives of the present study were to: (1) Determine the occurrence of damping-off and stem rot of cowpea in Benin and identification of the causal organism/s (Chapter 3); (2) Investigate etiology and environmental factors favouring the development of the diseases (Chapter 4); (3) determine genetic variation among *Sclerotium* isolates (Chapter 5); (4) Identify a rapid screening method in the laboratory for cowpea resistance to the disease (Chapter 6) and role of polyphenolics in resistance to the disease (Chapter 7) and; (5) Develop other control strategies to reduce the incidence of the diseases in the field (Chapters 8 and 9). During the current study, a survey was undertaken throughout the republic of Benin to determine the disease occurrence, its incidence and associated fungi. Results showed the existence of the disease syndromes in the five agro-ecological zones and the major causal agent throughout the country was *Sclerotium rolfsii* Sacc. Other minor pathogens were *Pythium* sp., *Rhizoctonia solani* Keulh and *Phoma pomorum* Thüm. Among factors influencing the disease incidence, cultural practices such as sole crop and no-till systems appeared to be most important. The highest incidence was recorded in the areas (South and Centre) with no-tillage coupled with sole cropping or plant residue burying before planting. *Sclerotium rolfsii* is a soilborne fungus and weeds and plant residues easily carried its initial inoculum. The cultural practices in the South and Centre created favourable conditions for *S. rolfsii*, and cowpea plants were directly exposed to the

existing initial inoculum which lead to rapid disease initiation (Singh and Rachie, 1985). In sole cropping, the pathogen easily spreads to nearby plants and as a result, the disease development increases and can lead to a higher incidence (Coakley, 1988) like that observed in the zones. Results from this current study provide the first documentation on the countrywide distribution of the diseases, the associated fungi and cultural practices that favour the disease incidence. Pending an appropriate and sustainable solution to the diseases, proposed control measures could include the gathering and burning of the infected plants at one place, where applicable, planting seeds on raised beds (till) with appropriate plant spacing, as suggested by Lucas *et al.* (1993). These measures would help reduce or eliminate the initial inoculum before planting and break the disease spread. These measures can be reinforced by the use of the combination of *M. oleifera* extract as a seed treatment and *Trichoderma* as soil treatment.

The survey results showed that the Ouémé valley (in the South zone of Benin) was the area with the highest disease incidence. The reason for this was not known. In the present study, particular attention was paid to this valley and some epidemiological aspects, such as initial inoculum, rainfall, air temperature, relative humidity and soil moisture were studied in Agonguey, Ouémé valley, and correlated to the disease incidence. The results indicated that soil moisture and particularly the *S. rolfsii* initial inoculum were the main factors influencing the disease incidence. The Ouémé valley is a zone where cowpea cultivation starts after natural flooding and farmers practice no-tillage coupled with cowpea as sole crop. The plant residues are left on the soil surface and allowed to rot after flooding. Each year, farmers, make rows in the residues in which the plant cowpea seeds are planted. As stipulated (Chapter 3), the initial inoculum is present at planting in this practice and ready to initiate disease. *Sclerotium rolfsii* grows fast and can multiply its inoculum throughout a season. Most of the inoculum (as sclerotia) would have constituted the initial inoculum for the following year and most likely successive years, leading to the high disease incidence found in the present study.

Within the valley, disease incidence varied from one site to another, though *S. rolfsii* was found to be the causal agent in the valley. Previous studies showed that *S. rolfsii* isolates from different sites in the valley showed variation in terms of growth rate, number and time to first production of sclerotia on potato dextrose agar (PDA) (Adandonon, 2000). A genetic population study of the *S. rolfsii* isolates collected from

the Ouémé valley, Benin and South Africa (and also reference isolates) was conducted, in order to determine the extent of possible existing variation among isolates. Aspects such as mycelial compatibility groups (MCG) and ITS rDNA sequence analysis were studied. Results showed two different MCGs (MCG 1 and MCG 2) through the valley. The two MCGs could be found in one site, or one MCG found in different sites. The finding of one MCG in different sites might be possible through dissemination by flooding water or cultural materials. The recovery of one or two MCGs in one site might explain the difference in disease incidences. The ITS rDNA analysis indicated that variation is present, but still low, compared to previous genetic analysis on the pathogen (Punja and Grogan, 1983; Harlton *et al.*, 1995; Cilliers *et al.*, 2000).

In the valley, farmers have noticed that the more a cultivar is cultivated, the greater the yield losses due to the diseases (PEDUNE-BENIN, 1995). They often complain about the diseases and frequently change cultivars as they become more susceptible. Thus, a quicker and easier method of screening cultivars against the pathogen infections, before cultivars are released and widely adopted, is necessary. In this regard, two rapid screening methods, namely on-paper and on-PDA methods were evaluated in the laboratory and data compared to the incidence of the disease at naturally infected field. The on-paper screening method data in the laboratory correlated well with the disease incidence recorded in the greenhouse and naturally infected soil. This finding is very important since the technique is very easy, helps obtain tolerant/resistant cultivar within 12 d and therefore helps farmers make a timely decision of which cultivars to plant.

Chemicals analysis conducted during the study supported the screening results and showed that phenolic compounds are involved in the tolerance of the cultivars. Tolerant cultivars have pre-existing phenolics which are higher than those in the susceptible ones. Forty-eight hours after inoculation, the tolerant cultivars produced two types of phytoalexins that were absent in the susceptible cultivars. Phytoalexins are known to be involved in the resistance process in plant (Latunde and Lucas, 2001) and their presence in this study might be the main factors explaining the behaviour of the cultivars after inoculation with the pathogen. This result of the phenolic study supported those of the on-paper rapid screening method in the laboratory and showed that phenolic compounds present before and after *S. rolfsii* infection, could also be used as screening parameters for resistance or tolerance of cowpea against *S. rolfsii* damping-off and stem rot.

So far, there are no control measures to reduce the yield losses inflicted by the diseases on cowpea in Benin. Considering the pressing need to improve the welfare of rural and urban poor population, issues relating to better food supply and greater opportunities for income generation are of paramount importance (Singh *et al.*, 1997). Therefore, appropriate control measures are necessary to efficiently combat the diseases as they can have serious economical implications. In addition to the rapid screening method developed, other control strategies have been evaluated in this study including the use of *M. oleifera* extracts, *Trichoderma* Kd 63, *Trichoderma* IITA 508 and *B. subtilis*, on their own or integrated, to control the diseases in the field. Results indicated that *M. oleifera* extract at a concentration of 15 kg/10 l water was effective as a cowpea seed treatment for adequate *Sclerotium* disease control in the field. Moreover, *M. oleifera* leaf extract seed treatment, on its own, significantly reduced disease incidence in the field experiment, as did both *Trichoderma* strains. A remarkable effect on the disease incidence and the best disease control was recorded when seed treatment with *M. oleifera* leaf extracts was combined with a soil sprinkle of *Trichoderma* Kd 63 and particularly *Trichoderma* IITA 508. These results could indicate the synergistical effect of *Trichoderma* and *M. oleifera* in the protection of the further growth of the plant against *S. rolfii* infection. Antagonistic (parasitic) effects of *Trichoderma* on many soilborne pathogens have been reported (Weidman and Wehner 1993) as has fungicidal effect of *M. oleifera* leaf extract in the protection of cereal and legume crops against soilborne diseases (SIBAT, 1993; Stoll, 1988). Early researchers also reported an increase in yield and in minimising environmental hazards when two or more control measures are applied (Papavizas, 1985; Pusey, 1994). *Moringa oleifera* is grown locally in Benin and its extracts are easy to prepare. Moreover, *Trichoderma* IITA 508 was collected from the naturally *Sclerotium*-infected field in the valley and culture of its inoculum on millet seed is easy. Therefore, farmers could quite easily adopt this integrated control (combination of *M. oleifera* and *Trichoderma*).

The present work provides with the first documentation on the presence of damping-off and stem rot and the threat the diseases represent for the cowpea cultivation throughout the Republic of Benin. Furthermore, a new rapid screening method was identified and will help select tolerant cultivars from a cultivar bank. The damping-off and stem rot management can be based on the use of resistant or tolerant cultivars, integrated with appropriate cultural practices, use of *M. oleifera* leaf extracts and *Trichoderma* soil

treatments. This integrated method will undoubtedly lead to an interesting ecologically sustainable cowpea production and protection in the field.

Although the current study yielded interesting results, various points still need to be understood. During the disease survey, farmers were not aware of the existence of the disease in their fields and considered the disease symptoms due to weeding injury. This points out the need for training of farmers. Moreover, future research in Benin should focus on the following:

- Study of the population genetics of *Sclerotium rolfsii* including as many isolates from different regions and various hosts in Benin, in order to reflect the full extent of genetic variation among isolates. This will be of paramount importance in breeding programmes
- Breeding of cowpea cultivars for resistance to *S. rolfsii* damping-off and stem rot, based on the genetic variability among isolates. The on-paper screening method developed in the present study could help in the rapid testing of the new cultivars.
- Characterisation and purification of the main biologically active ingredient of *M. oleifera* extract and identification of its mode of action, in order to increase the efficacy of the extracts in the field.

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## **SUMMARY**

SUMMARY

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In the Ouémé, Republic of Benin, damping-off and stem rot are the most important cowpea diseases resulting in up to 40 % yield losses and is therefore an important income loss for farmers. However, little is known about the disease distribution throughout the country.

In the field, damping-off symptoms observed included necrotic, water-soaked and brown lesions on the hypocotyl. The diseased seedlings showed wilting and fell over on the ground. When cowpea plants were affected at a later growth stage, the disease was called stem rot and the affected plants developed the above mentioned symptoms, but remained upright. Results from the disease survey throughout Benin showed that the disease syndromes and variable incidences were found in the five agro-ecological zones, emphasising the importance and widespread distribution of the diseases in the country. The highest incidence was recorded in the South and Centre zones of the country. This is the first report of the countrywide distribution and incidence of damping-off and stem rot of cowpea in Benin. Although *P. pomorum*, *Pythium* sp. and *R. solani* were found in different areas, *S. rolfsii* was the predominant fungus isolated and was considered as the major pathogen because of its frequency of isolation and isolate pathogenicity.

In the Ouémé valley, Benin, interaction among isolated fungi was evaluated and aspects of epidemiology, such as initial inoculum, rainfall, air temperature, relative humidity and soil moisture, were studied in a site (Agonguey) and data correlated to the disease incidence. *Sclerotium rolfsii* was the causal agent in the same complexes with *F. oxysporum* or *F. scirpi* and *T. harzianum*. When *S. rolfsii* isolates were paired with *Fusarium* spp. and incorporated into the soil, it resulted in significantly fewer diseased plants than when the *S. rolfsii* isolates were used on their own. However, when the *S. rolfsii* isolates were paired with *T. harzianum* or in any other combination including *T. harzianum* and incorporated into the soil, no cowpea damping-off or stem rot was recorded. The initial inoculum density of *S. rolfsii* and incidence of damping-off and stem rot recorded in the field was higher closer to the river and the lowest in the plots farthest from the river, and positively correlated with initial inoculum density ( $r = +0.71$ ;  $P < 0.05$ ) and soil moisture (%) ( $r = +0.59$ ;  $P < 0.05$ ).

A genetic diversity study of the *S. rolf sii* isolates collected from the Ouémé valley, Benin and South Africa was conducted, in order to determine the extent of possible existing variation among isolates. Aspects such as mycelial compatibility groups (MCG) and ITS rDNA sequence analysis were studied. Results showed two different MCGs (MCG 1 and MCG 2) through the valley. The ITS rDNA analysis showed a relatively low genetic diversity among isolates.

During the present study, different control strategies were developed including a rapid screening method for resistance to the pathogen, use of *M. oleifera* extracts, *Trichoderma* and *Bacillus* on their own, or integrated, to control the diseases in the field. In the screening evaluation, two different methods, namely a paper- and a PDA-based method, were developed. The paper-based screening method was the best method, yielding tolerant cultivars grouping that had the lowest disease incidence in the field. The chemical analysis showed that phenolic compounds were involved in the tolerance of the cultivars to the disease. Tolerant cultivars have pre-existing phenolics which were higher than those in the susceptible ones. Forty-eight hours after inoculation, the tolerant cultivars produced two types of phytoalexins that were absent in the susceptible cultivars.

*M. oleifera* leaf extracts, *Trichoderma* Kd 63, *Trichoderma* IITA 508 and *B. subtilis* were tested for their efficacy in the greenhouse and field. *Moringa oleifera* leaf extract seed treatment, significantly reduced disease incidence in the field experiment, as did *Trichoderma*. A remarkable effect on the disease incidence and the best disease control was recorded when a seed treatment with *M. oleifera* leaf extract was combined with a soil sprinkle of *Trichoderma* Kd 63 or *Trichoderma* IITA 508.

In the present study, we were able to gain significant insight into the presence of damping-off and stem rot and the threat the diseases represent to the cowpea cultivation throughout the Republic of Benin. It also proposes some control measures against the diseases caused by *S. rolf sii* in the field. Finally, the need for further research was highlighted by the current work.