

THE EPIDEMIOLOGY AND CONTROL OF CRUCIFER CHOCOLATE SPOT

by

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ABSTRACT

In the 1991/92 season, a leaf spotting disease of crucifer seedlings appeared at a number of nurseries in Kwazulu-Natal (KZN) Province. Towards the end of 1996, the disease had also been detected in Western and Northern Provinces, and even in Zambia.

The disease appeared in nurseries during warm humid conditions. It initially started as small, water-soaked spots on leaves, which later expanded and coalesced, forming halo regions surrounded by a dark brown to black ring. Due to the typical dark brown spots, the disease was named crucifer chocolate spot (CCS). From the studies conducted by Qhobela & Laing (1994), the causal agent of CCS was found to be a *Xanthomonas campestris* pathovar that was in symbiotic association with a unique bacteriophage.

Koch's postulates were conducted. The causal agent of CCS was inoculated on cabbage seedlings (6-8 weeks old), and the seedlings subjected to post-inoculation periods of approximately 48 hours at 28-30°C, and relative humidity (RH) of ≥70%. The best method of inoculation was achieved when the leaves were pricked with pins prior to application of the inoculum ($\geq 10^7$ cfu/ml). The latent period of CCS varied from 2-6 days, depending on RH, temperature, nutritional status of the host and other factors within the crop pathosystem.

Artificial inoculation studies showed the host range of CCS *X. campestris* to be similar to *X. campestris* pv. *armoraciae* and *X. campestris* pv. *campestris*. All the pathogens were able to infect cabbage, cauliflower, broccoli and lettuce plants during artificial inoculation.

A hypersensitive response was elicited on pepper, tomato, and tobacco plants.

Electron microscopic studies showed epiphytic colonization by CCS *X. campestris*, and also the colonization of xylem, phloem and parenchyma tissues. Although xylem tissues were also colonized, the V-shaped lesions and vein blackening characteristics typical of *X. campestris* pv. *campestris* were not observed. Profuse multiplication of the pathogen resulted in rupturing of cell organelles and cell membranes.

Different crucifer cultivars from two seed companies were found to have varying levels of susceptibility to CCS disease during artificial inoculation, with levels of leaf area infected ranging from 0% to 34%. Disease severity was also affected by seasonal variations.

None of the seed-lots tested in this research were found to be infected by CCS *X. campestris*, although some were infected by other xanthomonads. Chemical seed treatment (soaking in bactericide solutions at 30°C for two hours) with biocides; e.g., Kocide 101, copper oxychloride, and copper ammonium carbonate was more effective than hot water treatment (50°C for 30 min). All seed treatments lowered pathogen levels, however, none resulted in complete control of seedborne inoculum. Percentage germination of seed was low for chemical treatment compared to the hot water treatment.

Disease levels were reduced with increased rates of Ocean 3.1.3 (38) fertilizer applied, with significant differences in the percentage leaf area infected at obtained at application levels of 400 ppm. Increased levels of N, P, K, Zn and Cu in leaf tissues were associated with low levels of disease severity.

Copper bactericides; e.g., Kocide 101, Copper Count N, Copper Ammonium Carbonate B and Copper oxychloride were more effective than quaternary ammonium compounds in reducing disease levels ($\leq 30\%$). The combination of copper bactericides and mancozeb improved the performance of the copper compounds. Application of copper bactericides up to four times the recommended dosage did not result in increased efficacy.

CCS was found in 80% of KZN nurseries surveyed between December 1995 and December 1996. During this period, it was not detected from Mpumalanga and Northern Provinces. Factors that contributed to CCS disease outbreaks include favourable climatic conditions ($\geq 40\%$ RH, $\geq 24^{\circ}\text{C}$), wounding of seedlings by pests, and nutrient stress.

DECLARATION

I, Kulani Patrick Machaba, declare that this thesis, except where otherwise indicated, is my own original research. It has not been submitted in part or as a whole, for a degree at any other university.

A handwritten signature in black ink, appearing to read "K. P. Machaba". The signature is written in a cursive style with a small flourish at the end.

K.P. Machaba

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CHAPTER 1. GENERAL INTRODUCTION

The family Cruciferae has 180 genera, and approximately 2000 species (Siemonsma & Piluek, 1993). Brassicas (part of the Crucifer family) are native to the Eurasian continent, and they are all grown from seed (Siemonsma & Piluek, 1993). Cabbage (*Brassica oleracea* var. *capitata* L.) is an important horticultural crop throughout South Africa, where there is a substantial market for this vegetable (Table 1.1. and 1.2.).

Production of crucifer seedlings in South Africa is mainly through the use of the Speedling® system in commercial nurseries (Laing, 1996). In the 1991/92 season, a leaf spotting disease of crucifers appeared at a number of seedling nurseries in Kwazulu-Natal Province. This disease caused serious economic losses and it made the future of cabbage seedling production bleak for a number of seedling growers. Characteristic symptoms are dark brown to black water-soaked spots, which spread in typical bacterial fashion and later coalesce forming halo regions (Laing & Qhobela, 1995). Due to the typical dark spots surrounded by a chocolate-coloured ring, the disease was named crucifer chocolate spot (CCS) (Laing & Qhobela, 1995).

There are chocolate spot diseases of other crops:

- i) Chocolate spot of maize (*Zea mays* L.), caused by *Pseudomonas coronafaciens* pv. *zeae* (Smith) (Nyvall, 1989).
- ii) Chocolate spot of beans (*Phaseolus vulgaris* L.), caused by the fungus *Botrytis fabae* Pers. ex Fr. (Hanounik & Robertson, 1988).
- iii) Chocolate spot of papaws (*Papaya caricae* L.), caused by the fungus *Colletotrichum gleosporioides* (Penz.) Penz. & Sacc. (Alvarez & Nishijima, 1987).

Phenotypic studies initially showed the causal agent of CCS to belong in the *Xanthomonas campestris* (Pammel) Dowson group, phenon 9 (Kariem *et al.*, 1995). However, further probing by the use of fatty acid methyl ester analysis, SDS-PAGE profiles of whole-cell proteins, restriction endonuclease digests of 16S rRNA PCR products and DNA fingerprinting using AFLP showed the CCS isolates to represent a distinct group from *X. campestris* pv. *campestris* (Kariem *et al.*, 1995). SDS-PAGE profiles of whole cell-proteins showed the isolated strains to be substantially different from known *X. campestris* pathotypes, namely, *X.c.* pv. *aberrans* (Knosel) Dye, *X.c.* pv. *armoraciae* (McCulloch) Dye, *X.c.* pv. *campestris* and *X.c.* pv. *raphani* (White) Dye (Kariem *et al.*, 1995).

Based on the results of characterization tests, two theories arose concerning the taxonomic state of the causal agent of CCS. The first theory is that the causal agent constitutes a strain variant of the pathovar *X.c.* pv. *campestris* (Qhobela¹, 1997: pers. comm.). The second theory is that the causal agent constitutes a new pathovar (Laing², 1997: pers. comm.). Factors mitigating against the former theory are that the epidemiology and symptoms of CCS and black rot (caused by *X.c.* pv. *campestris*) are substantially different (Figs 1.1 & 1.2; Table 1.3.). Factors mitigating against the latter theory are that the restriction edonuclease digest of the 16S rRNA gene of this pathogen is indistinguishable from that of *X. c.* pv. *campestris*, and the host range of this organism is similar to that of *X. c.* pv. *campestris* during artificial inoculation (Kariem *et al.*, 1995). The former theory accommodates a classification system based on a single phenotypic feature; i.e., host-specificity. However, current classification of all phenotypically indistinguishable nomenspecies as pathovars of *X. campestris* is generally considered to be a compromise between the viewpoints of plant pathologists on the one hand, who need names for bacteria that are pathogenic to specific hosts, and bacterial taxonomists on the other hand, who require differentiating phenotypic features for the description of species (Vauterin *et al.*, 1993). As the taxonomy of *Xanthomonas* progresses, it is obvious that the current classification is not a reflection of real relationships within the genus (Vauterin *et al.*, 1993; Vauterin *et al.*, 1995). These differences emphasize the need to review the taxonomy of this heterogenous pathovar (Qhobela¹, 1997: pers. comm.).

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Xanthomonas infections occur on at least 124 monocotyledonous and 268 dicotyledonous plant species (Leyns *et al.*, 1984). Although xanthomonads are plant-associated bacteria, there are some *Xanthomonas campestris* isolates that occur in asymptomatic association with plant tissues or as epiphytes (Maas *et al.*, 1985). Other xanthomonads appear to be limited to secondary invasion of plant tissue after infection by a primary bacterial pathogen (Gitaitis *et al.*, 1987; Hayward, 1993).

Crucifer seed lots tested in the initial stages of disease outbreaks were found to be contaminated by the CCS causal agent (Laing², 1996: pers. comm.). Seed is an important source of *X. campestris* inoculum (Schaad *et al.*, 1980, Shekhawat *et al.*, 1982). Xanthomonads are associated with seed as an infection or an infestation (Agarwal & Sinclair, 1987b).

Several laboratory assays are available for detecting *X. campestris* in infected crucifer seed. Such assays must fulfil the following requirements:

- i) They must give reliable information pertaining to field performance and quarantine requirements (Agarwal & Sinclair, 1987a; Neergaard, 1977).
- ii) They must provide safeguards against the recording of false positives (Maude, 1996).
- iii) They must be sensitive (detect causative organisms at low numbers) (Maude, 1996).
- iv) The time, labour and equipment for carrying through the test must be kept within economic limits (Neergaard, 1977).
- v) The tests must be reliable and reproducible within statistical limits (Neergaard, 1977; Maude, 1996).
- vi) The methodology should be simple to enable the tests to be performed by not necessarily highly qualified persons (Maude, 1996).

In addition to seed-borne inoculum, other factors that may affect the epidemiology of xanthomonads include free water, RH, temperature, nutritional status of the host, wind, insects, weeds and debris (Schaad & Dianese, 1981; Schaad, 1982; Goto, 1992; Zehr *et al.*, 1996).

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The research project on CCS disease had two phases. The first phase, which encompassed a study of the CCS disease epidemiology and control, was conducted at the University of Natal. The second phase, which involved a comprehensive taxonomic study of the CCS putative pathogen, was conducted at the University of Cape Town. This thesis is based on trials conducted to elucidate the epidemiology of CCS and to develop an integrated disease control strategy for nurserymen in South Africa.

Table 1.1 Value of crops in lands, South Africa (Department of Agriculture, 1994)

Crop	Price (Rand/ ton)	Ave. yield/ ha	Gross value/ ha	Net Profit/ ha	Max. Yield/ ha	Max. Profit/ha
Maize	535	4t	R2 140	R800	12t	R2 400
Wheat	800	3t	R2 400	R1 200	6t	R2 000
Cabbage	240	50t	R12 000	R7 000	120t	R16 800
Tomato	1000	40t	R40 000	R20 000	120t	R60 000

Table 1.2 Vegetable production in South Africa from 1980-1993 (Department of Agriculture, 1994)

Crop	Production x 1000t			Price R/t		
	1980	1990	1993	1980	1990	1993
Potatoes	259	1270	1127	136	510	968
Tomatoes	310	389	422	345	901	1000
Onions	142	208	249	224	543	872
Pumpkins	141	167	177	115	359	363
Cabbage	225	212	241	58	180	240
Sweet Potatoes	45	54	59	182	401	474
Cauliflower	48	41	37	93	270	314
Beans	32	30	28	356	1219	1436
Beetroot	43	32	34	128	409	436
Peas	23	24	20	283	1085	1652
Carrots	107	40	94	135	452	455

Table 1.3 Similarities and differences between chocolate spot and black rot diseases of crucifers

Characteristics	Chocolate spot	Black rot
Symptoms	Small water-soaked spots in the middle of leaves of stressed seedlings. Blackening of veins not seen except when occurring together with black rot. No chlorosis.	Small, V-shaped lesions on the edges of leaves of seedlings or heading plants. Blackening of the veins of leaves and chlorosis usually occurs.
Plants infected	Mostly crucifer seedlings. Rarely seen in the field.	Mostly heading plants in the field. Seedlings can also be infected.
The role of stress	Disease more severe when seedlings are stressed.	Disease prevalent also in seedlings that are not highly stressed.
Environmental requirements	Both infect plants in conditions of high RH and temperatures ranging from 22-30°C.	
Hypersensitive response	Both cause hypersensitive responses on tomato, pepper and tobacco plants	
Infection pathway	Mostly via wounds and stomata. Fails to infect via hydathodes.	Mostly via hydathodes. Can also infect via wounds and stomata.
Colony morphology	Young colonies are slightly yellow and mucoid. Old colonies are deep yellow in colour.	Young colonies are yellow and mucoid. Old colonies have a deep yellow to orange colour.
Important source of inoculum	Seedborne inoculum very important in both diseases.	
Severity	Not very important on mature plants.	Very severe on mature plants. Not very severe on young seedlings.



Fig 1.1 Leaf symptoms of crucifer chocolate spot disease on a cabbage seedling



Fig 1.2 Leaf symptoms of black rot disease on a cabbage seedling

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CHAPTER 2. OPTIMIZING CONDITIONS FOR ISOLATION AND INOCULATION OF THE CAUSAL ORGANISM OF CRUCIFER CHOCOLATE SPOT

Abstract

Different methods of inoculum preparation and inoculation techniques were evaluated for the crucifer chocolate spot-associated *Xanthomonas campestris*. Inoculum concentrations of $>10^8$ cfu/ml were needed for the development of symptoms of crucifer chocolate spot disease. Wounding of leaves by pricking with multineedles was found to be the best method of inoculation. Washed cells gave improved levels of infection compared to the unwashed cells, possibly because the treatment reduced the amount of slime in the inoculum.

2.1. Introduction

Crucifer chocolate spot (CCS) is caused by a *Xanthomonas campestris* pathovar, which is different from other known xanthomonads (Kariem *et al.*, 1995; Laing & Qhobela, 1995). Bacteria belonging to the genus *Xanthomonas* are plant-associated bacteria and they are not usually encountered in other environments (Hayward, 1993). Isolates resembling *X. campestris* pv. *campestris* have been found in asymptomatic association with plant tissue or as epiphytes (Maas *et al.*, 1985), and there are other xanthomonads which appear to be limited to secondary invasion of plant tissue after infection by a primary bacterial pathogen (Gitatiti *et al.*, 1987; Hayward, 1993). Epiphytic populations of xanthomonads do not necessarily indicate epiphytic growth because they can reflect bacterial exudation from lesions (Rudolph, 1993).

Pathogenicity tests aid in determining the identity and virulence of xanthomonad isolates. For pathogenicity tests to be successful, conditions favouring disease development have to be determined. In this study, Koch's postulates were performed to verify the assumption that an organism isolated in pure culture was the actual cause of CCS.

2.2 Materials and Methods

Isolation of the causal organism

The organism was isolated from diseased cabbage (*Brassica oleracea* var. *capitata* cv. Green Coronet) seedling leaves by cutting out the border of diseased and healthy tissues and surface sterilizing in 0.35% sodium hypochlorite for three min, followed by rinsing twice in sterile distilled water. A sterile mortar and pestle was used to grind the excised tissue, followed by streaking onto agar media. The media used during the isolation process were nutrient agar (NA), nutrient starch cycloheximide antibiotic agar (NSCAA) medium (Randhawa & Schaad, 1984), starch *Xanthomonas* (SX) medium (Schaad & White, 1974) and *Xanthomonas campestris* pv. *pruni* (XPS) medium (Civerolo *et al.*, 1982). Plates were incubated for 48 h at 28°C. After 48 h, pure cultures were obtained by two transfers (triple streaking) on NA.

Preparation of the inoculum

Two loopfulls of growth from 36-48 h old cultures on nutrient agar medium were transferred to 75 ml nutrient broth in 250 ml Erlenmeyer flasks. The flasks were shaker incubated (150 rpm) at 30°C for 36-48 h. The cells were then pelleted by centrifuging at 3000g for 10 mins. The supernatant was decanted and the pellet resuspended in sterile Ringer's Solution. The concentration of the cells was adjusted to 10^4 , 10^8 and 10^{10} cfu/ml using OD_{620} values calculated (Table 2.1). Cell concentrations were checked by plate counts, with three replicates per dilution. In a second method, the cells were not centrifuged, but were inoculated at similarly adjusted concentrations immediately after culturing for 48 h in nutrient broth.

Test Plants

Six to seven week old cabbage seedlings (cv. Green Star) grown in composted pine bark medium were obtained from a nursery in size 200 Speedling® trays and transferred to size 24 Speedling® trays. The seedlings were irrigated for a week without any fertilizer, and thereafter inoculated.

Inoculations were made using six different techniques:

1. Pricking the upper part of young leaves and petioles with a device consisting of 10 pins mounted in a cork. The plants were then sprayed with the inoculum by the use of an atomizer (Gupta & Chakravarti, 1983)
2. Puncturing the upper part of petioles and leaves with the above device and thereafter rubbing the inoculum onto damaged areas with a wad of cotton wool which had been dipped into the inoculum
3. A syringe fitted with a 26 gauge needle was used to inject the leaves with the inoculum (Schaad, 1988)
4. A sterile cocktail stick dipped in the inoculum to inoculate the petioles and main veins of young leaves
5. Rubbing carborundum powder onto the leaves and then rubbing over the injured area with a wad of cotton wool dipped in the inoculum
6. Sterile scissors to cut the leaves of seedlings and then passing a cocktail stick dipped in the inoculum along the edges of the cut leaves (Chen *et al.*, 1994)

The plants were subsequently moved to a dew chamber held at 80-100% RH and a temperature of 28-30°C for 48-72 h (Zehr *et al.*, 1996). The inoculated seedlings were then left in the dew chamber (12 h photoperiods) for 48 h. After 48 h, the plants were moved into the glasshouse where the temperature was 27-30°C and RH was 50-60%.

2.3 Results

Table 2.1 Number of cfu/ml of CCS *X. campestris* at different dilutions

Dilution	Number of colonies/plate	Number of cfu/ml	OD ₆₂₀
10 ⁻²	102	1.02 x 10 ⁴	0.328
10 ⁻³	97	9.7 x 10 ⁴	0.273
10 ⁻³	89	8.9 x 10 ⁴	0.262
10 ⁻⁷	52	5.2 x 10 ⁸	0.191
10 ⁻⁷	41	4.1 x 10 ⁸	0.180
10 ⁻⁷	38	3.8 x 10 ⁸	0.187
10 ⁻¹⁰	<30	-	0.088
10 ⁻⁹	30	3.0 x 10 ¹⁰	0.097
10 ⁻⁹	33	3.3 x 10 ¹⁰	0.104

Table 2.2 Pathogenicity of CCS *X. campestris* to cabbage seedlings following inoculation using different techniques

Inoculation technique	Concentration of washed bacterial cells (cfu/ml)			Concentration of unwashed cells (cfu/ml)		
	10 ⁴	10 ⁸	10 ¹⁰	10 ⁴	10 ⁸	10 ¹⁰
Pin-pricking leaves then spraying inoculum	Fair	Good	Good	Poor	Fair	Good
Pin-pricking leaves then rubbing in inoculum using cotton wool	Poor	Good	Good	Poor	Fair	Good
Injection of inoculum using a syringe fitted with a needle	None	Fair	Fair	None	Poor	Poor
Inoculation of main veins of leaflets using a cocktail stick dipped in the inoculum	None	Poor	Fair	None	Poor	Poor
Rubbing carborundum onto the leaves then rubbing in the inoculum	None	Fair	Fair	None	Poor	Poor
Inoculating the cut edges of leaves severed with a pair of scissors	None	None	Poor	None	None	None

None: No infections

Poor: Few infections

Fair: Infections inconsistent

Good: Infections consistent

The isolated organism produced colonies that were yellow, mucoid and shiny, and they did not attach easily to the loop during streaking. There was no growth on SX medium during isolation from diseased plant material. The recovery of saprophytic bacteria was high on nutrient agar, and this made the distinction between *Xanthomonas campestris* colonies and other yellow pigmented bacteria difficult. OD₆₂₀ values of the bacterial cells ranged from 0.104 to 0.328 for 3.3×10^{10} cfu/ml to 1.02×10^4 cfu/ml respectively (Table 2.1).

Following inoculation, significant disease levels were obtained at inoculum concentrations of 10^8 and 10^{10} cfu/ml. The symptoms were black to dark brown spots, sometimes with slight chlorosis around the inoculated areas. Poor infection was obtained when the unwashed cells were used during inoculation.

During pathogenicity tests, Methods 1 and 2; i.e., puncturing the leaves and petioles with pins and then applying the inoculum, were found to be the best methods of inoculating the CCS organism into seedlings of crucifers. When the other inoculation methods were used, the degree of infection varied (Table 2.2).

2.4 Discussion

The variation in the amount of infection obtained when unwashed cells were used as the inoculum may be attributed to the slimy material released by the xanthomonad cells. As *X. campestris* produces xanthan gum (Goto, 1992), this could lead to blockage of the wounds on the damaged areas of the mesophyll, resulting in poor penetration of the host cells/tissues by the bacteria (Wallis *et al.*, 1973). Washed cells had little slime, thereby possibly enhancing the penetration of wounded tissue.

The high relative humidity obtainable in the dew chamber resulted in higher infection rates. Maintenance of leaves in saturated conditions could lead to anoxia and this may result in an increase in the permeability of plant cell membranes, a fact which accounts for the faint chlorosis which often develops after water-logging with sterile water (Young, 1974).

Yellowing during artificial inoculation may also result from toxins or anti-bacterial compounds released by the plant as part of the defence mechanisms.

The limitation of the pin-pricking inoculation technique is that it can produce false positive results. False positive results appear when the plants are highly stressed and thus show disease symptoms incited by any inoculated pathogen as a result of the stress and not susceptibility *per se* (Goto, 1992).

Certain techniques for the introduction of bacteria into plants can give rise to symptoms of disease, even though bacterial isolates which are not normally pathogenic to crucifers are used (Robeson *et al.*, 1989). However, in the present case the causal agent of CCS infects plants when they are stressed, wounded (insect bites) or when there is some physiological disorder within the infected plants. For example, excessive irrigation or rain in seedling nurseries leach nutrients in the vicinity of the roots, resulting in stressing of the crucifer seedlings thereby increasing their vulnerability to CCS.

Initiation of disease by phytopathogenic bacteria is dependant on the concentration of the inoculum, and the minimum concentration needed for the development of *X.c. pv. campestris* is commonly in the region of 10^6 cfu/ml (Staub & Williams, 1972, Gupta & Chakravarti, 1983). In this study, plants were also found to be more susceptible to high inoculum levels of the CCS bacterium.

The putative pathogen was found to be the causal agent of CCS, thus satisfying the requirement of Koch's postulates.

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CHAPTER 3. EFFECTS OF ENVIRONMENTAL FACTORS ON *IN VIVO* CRUCIFER CHOCOLATE SPOT DISEASE INITIATION AND SEVERITY

Abstract

The effects of temperature and relative humidity on crucifer chocolate spot disease were determined. The optimum temperature for the expression of symptoms of the disease was 28°C. Symptom initiation was faster (48-72 h) at 80-100% relative humidity (RH) than at 50-70% RH (5-7 days). The latent period of crucifer chocolate spot disease varied from 2-6 days depending on the RH, temperature, and other factors within the crop pathosystem.

3.1 Introduction

Epidemics of plant diseases result from the interaction of processes at three system levels: organismal processes (plant and fungal), population processes (crops and pathogens) and community processes (ecosystems) (Zadocks & Schein, 1979). The multiplication of pathogenic bacteria within plant tissues can be affected by temperature, humidity, wind, sunlight and other environmental factors (Goto, 1990). Some xanthomonad incited diseases that are severe at 25-30°C are *Xanthomonas campestris* pv. *oryzae* (Ishiyama) Dye, *X.c.* pv. *carotae* (Kendrick) Dye and *X.c.* pv. *pruni* (Smith) Dye (Goto, 1990; Zehr *et al.*, 1996).

In plants, water congestion, defined as the accumulation of excessive water in the intercellular spaces as a result of internal water pressures, and leaf wetness are prerequisites for development of bacterial spot of peach (*Prunus persicae* L.) caused by *X.c.* pv. *pruni* (Zehr *et al.*, 1996). RH affects the duration of water congestion, amount of bacterial exudation, epiphytic populations of pathogens, and interactions between pathogens and saprophytic microorganisms on plant surfaces (Goto, 1990).

Crucifer chocolate spot (CCS) is caused by a *Xanthomonas campestris* pathovar and the objective of this experiment was to assess the effect of temperature on the percentage leaf area infected and the effect of six RH regimes on the initiation of symptoms.

3.2 Materials and Methods

3.2.1 Effect of temperature on percentage leaf area infected

Inoculum (10^8 cfu/ml) of the CCS pathogen (isolate PMK 9608, from Dr Qhobela of the University of Cape Town) was prepared in sterile diluted Ringer's Solution (as in 2.2). Cabbage seedlings (*Brassica oleracea* var. *capitata* L.) cv. Green Star (lot 340301/07030) growing in Speedling® 24 trays were kept in the greenhouse and fertilised with 100 ppm Ocean 3.1.3 (38) fertilizer until 7-8 weeks old. Inoculation was by pin-pricking the leaves of seedlings and thereafter rubbing inoculum into the wounds with a wad of sterile cotton wool. Ten plants per tray were inoculated at random (four replicates/treatment). The trays were then placed in growth chambers at 100% RH and temperatures of 20, 24, 28, 32 and 36°C. The control was inoculated with water. Percentage leaf area infected was estimated every five days from a visual rating scale (Appendix 1). The results were transformed into the AUDPC format (area under the disease progress curve) and analysed by ANOVA (analysis of variance). AUDPC is the integral over time of the percentage of foliage diseased, and was calculated through trapezoidal integration by using a computer program in Microsoft Basic (Berger, 1989).

3.2.2 Effect of RH on disease initiation

Infection of plants by the CCS pathogen was studied at six levels of RH and a constant temperature of 28°C. Cabbage seedlings (7-8 weeks-old) were inoculated (as in 3.2.1) and placed in Growth chambers. The relative humidity of the six Growth chambers was adjusted to 50, 60, 70, 80, 90 and 100% respectively. In all cases, a photoperiod of 12 h was used. The experiment was repeated three times.

3.3 Results

3.3.1 Effect of temperature on percentage leaf area infected

The greatest AUDPC value was obtained at 28°C, and the lowest at 20°C. The pathogen failed to infect and establish significant levels of disease at 36°C (Fig 3.1).

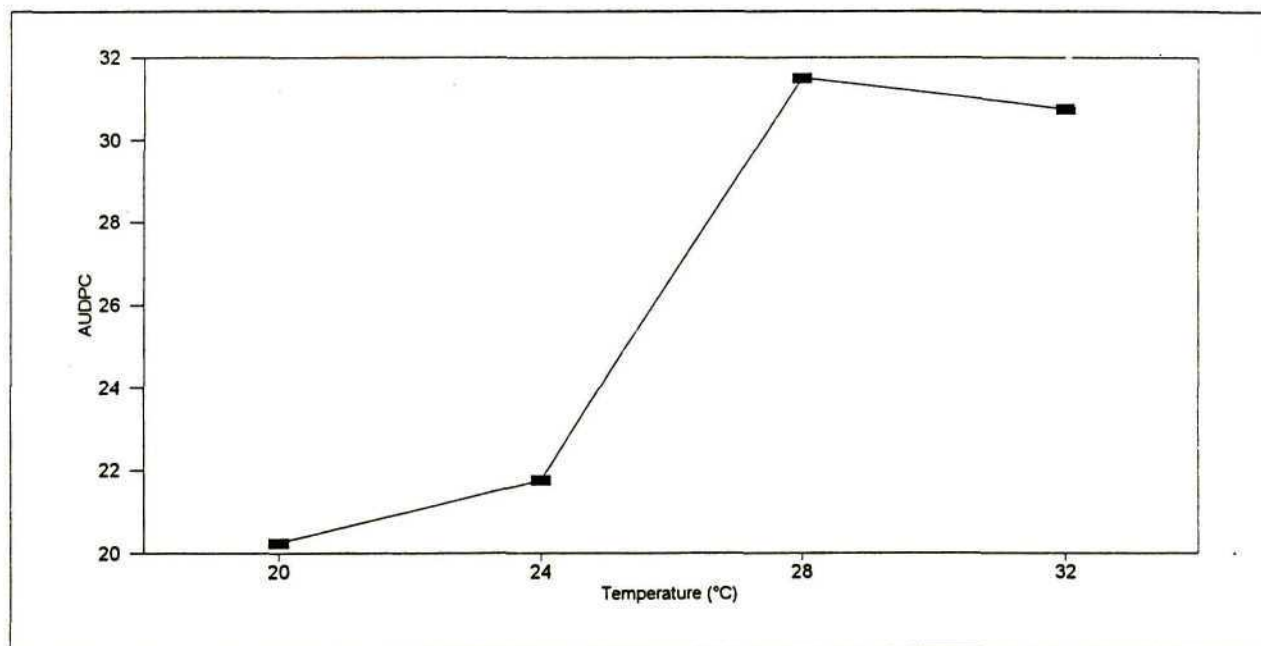


Fig 3.1 Area under the disease progress curve (AUDPC) of crucifer chocolate spot at different temperatures

3.3.2 Effect of RH on disease initiation

Symptom development was fast at RH of 80-100%, with post-inoculation periods of 48-72 h necessary before initial symptom expression in the growth chamber. At 50-70% RH, the plants had to stay in the growth chamber for 5-7 days before symptoms appeared.

3.4 Discussion

The highest AUDPC values for CCS disease were obtained at 28°C, and conformed to the range of 24-31°C reported by Zehr *et al.* (1996) for xanthomonad diseases. This differed slightly from the results of Staub & Williams (1972), who estimated the optimum temperature of *X. campestris* pv. *campestris*, causal organism of black rot, to be 30-31°C.

In nursery situations, outbreaks of CCS occur over the temperature range 24-30°C (refer to the Disease Survey - Chapter 9). However, it is possible that in the field infection occurs at low temperatures (20-24°C), whereas symptom expression requires warmer temperatures (24-27°C). Similarly, even though the pathogen might colonise the plants at low RH, the latter possibly remain symptomless for a few days before symptoms are expressed.

The latent period of CCS can be extrapolated to be 2-6 days depending on the RH, temperature, and other factors within the crop pathosystem. The knowledge of favourable temperatures and RH for the progress of CCS can help growers to predict periods favourable for outbreaks, and therefore introduce integrated management options (refer to Chapter 11). It also explains the consistent outbreak of this disease in nurseries where humid environments and warm temperatures prevail.

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CHAPTER 4. INFECTIVITY OF THE CAUSAL ORGANISM OF CRUCIFER CHOCOLATE SPOT ON CRUCIFEROUS AND NON CRUCIFEROUS PLANTS

Abstract

The host range of the crucifer chocolate spot causal agent was not different to that of *Xanthomonas campestris* pv. *armoraciae*, *X.c.* pv. *campestris* and *X.c.* pv. *raphani*. All these pathogens incited a hypersensitive response on pepper, tomato and tobacco plants, and all of the pathogens infected cabbage, cauliflower, broccoli, brussels sprouts and lettuce following artificial inoculation.

4.1 Introduction

Approximately 125 plant pathologically distinct xanthomonads are recognized, but few of these can be easily distinguished from the type species, *Xanthomonas campestris* (Pammel) Dowson or each other by the usual biochemical tests used in Bergey's Manual (Schaad, 1988). Historically, a number of species were identified as *Xanthomonas*, with no critical comparison of the causal organisms involved (Dye, 1962). Lists of cultural and biochemical characters are available but these lists are inconsistent, and give rise to doubt concerning the validity of the differences listed; some may present no more than natural variation among strains, while others could be due to differences in methods of testing, or of recording results used by different workers (Dye, 1962).

Contemporary methods used in the differentiation of xanthomonads to the pathovar level involve a combination of morphological, physiological, biochemical and serological techniques. Some pathovars might differ from one another by only one or a few virulence genes that have a high selective value on specific crop genotypes (Chen *et al.*, 1994).

In all (bacterial) plant diseases, four genes are involved in interaction of the microbe and the plant (Kamoun & Kado, 1990):

1. *hrp* (hypersensitive, response and pathogenicity) - is required for pathogenicity on hosts and for inciting hypersensitive response (HR) on non-hosts
2. *dsp* (disease specific) - is necessary for pathogenicity but do not affect HR induction on non-host plants
3. *avr* (avirulent) - confers race or cultivar specific relations to cultivars that contain complementary resistant genes
4. *hsv* (host specific-virulence) -is required for pathogenicity on non-host plants, and can extend the host range when introduced in narrow-host-range strains

Pathovar status can be determined either by genes that determine host range or by those that determine distinct phenotypes. Both types of genes should have a high selective value for the pathogen on its hosts and help determine a clonal population structure (Chen *et al.*, 1994).

The objective of this study was to determine the host range of the causal organism of crucifer chocolate spot (CCS), and to compare it with the host range of *Xanthomonas campestris* pv. *campestris*, *X.c.* pv. *armoraciae* (McCulloch) Dye and *X.c.* pv. *raphani* (White) Dye.

4.2 Materials and Methods

Test plants

The seeds were sown in size 24 Speedling® trays. Three different cultivars each from cabbage, cauliflower, broccoli, brussels sprouts, pepper, tomato and lettuce were used (Table 4.1). The seedlings were grown in a greenhouse and inoculated when they were 5-6 weeks old.

Table 4.1 Cultivars used during the host range trial

Common name	Latin name	Cultivars		
Broccoli	<i>B. oleracea</i> var. <i>italica</i> L.	Green Valiant	Kompacta	Pinnacle
Cauliflower	<i>B. oleracea</i> var. <i>botrytis</i> L.	Incline	Star 4401	Wallaby
Cabbage	<i>Brassica oleracea</i> var. <i>capitata</i> L.	Green Star	Hercules	Star 3301
Lettuce	<i>Lactuca sativa</i> L.	Commander	Star 5001	Summertime
Tomato	<i>Lycopersicon esculentum</i> L.	Karino	Zeal	Zest
Pepper	<i>Capsicum annum</i> L.	California Wonder	Jupiter	Komati

Preparation of the inoculum

Four different pathogens were used:

1. Three strains of the causal agent of CCS: PMK 9605, PMK 9608, and SA2
2. *X.c. pv. raphani* NCPPB 1946^T
3. *X.c. pv. armoraciae* NCPPB 347^T
4. *X.c. pv. campestris* NCPPB 528^T

The inoculum was prepared by transferring bacterial cells from a yeast dextrose carbonate (YDC) medium (Schaad, 1988) into nutrient broth. The cells were incubated for 36 h on a shaker (150 rpm) at 30°C. The cells were then harvested, centrifuged at 7000g and resuspended in Ringer's Solution. The concentration of the inoculum was adjusted to be around 10⁸ cfu/ml.

4.3 Results

Table 4.1 Pathogenicity of different *Xanthomonas* pathogens to various crops

Host/ Cultivar	XCA	XCC	XCR	PMK 9608	PMK 9605	SA2
1. Kompacta	+	+	+	+	+	+
1. Green Valiant	+	+	+	+	+	+
1. Star 4401	+	+	+	+	+	+
2. Star 3301	+	+	+	+	+	+
2. Wallaby	+	+	+	+	+	+
2. Incline	+	+	+	+	+	+
3. Pinnacle	+	+	+	+	+	+
3. Green Star	+	+	+	+	+	+
3. Hercules	+	+	+	+	+	+
4. Summertime	+	+	+	+	+	+
4. Commander	+	+	+	+	+	+
4. Star 5001	+	+	+	+	+	+
5. Zest	+	+	+	+	+	+
5. Karno	+	+	+	+	-	+
5. Zeal	+	+	+	+	+	-
6. California Wonder	+	+	-	-	-	-
6. Komati	+	+	+	-	-	-
6. Jupiter	+	-	+	-	-	-

+ : Positive pathogenicity

- : No symptoms

1 - Broccoli, 2 - Cauliflower, 3 - cabbage, 4 - lettuce, 5 - tomato, 6 - pepper

XCA - *Xanthomonas campestris* pv. *armoraciae*, XCC - *X.campestris* pv. *campestris*,

XCR - *Xanthomonas campestris* pv. *raphani*

PMK 9608, PMK9605, SA2 - Strains of CCS *X. campestris*

Symptoms started appearing 10-12 days after inoculation. Table 4.2 shows the results of the host range pathogenicity tests. There was little difference in the nature of symptoms incited by CCS *X. campestris*, *X.c. pv. armoraciae* and *X.c. pv. raphani* on plants of the crucifer family. CCS *X. campestris*, *X.c. pv. armoraciae* and *X.c. pv. raphani* induced water soaked spots (3-6mm in diameter), surrounded by dark brown to black rings which later dried and produced a halo region. *X.c. pv. campestris* caused systemic yellowing in some plants, accompanied by the typical V-shaped lesions.

All the isolates tested induced similar symptoms on lettuce plants. Dark brown to black, soft rot lesions, developed which subsequently spread from the point of inoculation to other areas of the leaves.

Tomato and pepper plants inoculated with *X.c. pv. armoraciae*, *X.c. pv. raphani* and *X.c. pv. campestris* developed brown spots which quickly dried, sometimes with a slight yellowing around the inoculated areas. CCS *X. campestris* produced similar symptoms on tomato plants, but the reproduction of these were inconsistent in pepper plants.

Strain PMK 9608 appeared to be highly virulent compared to strain PMK 9605 and SA2. Strain SA2 produced the weakest response in all the cultivars tested. All plants were not equally susceptible to these strains and symptom development on the cabbage cultivar, Green Star, and the broccoli cultivar, Pinnacle was poor.

The leaf panels of tobacco plants, inoculated with the causal agent of CCS or with *X.c. pv. campestris* became brown after one day, and seemed to be necrotic after two days. In the areas inoculated with *Pseudomonas* sp., there was shrinkage of the lamina after 4-5 days of inoculation. Leaf panels inoculated with Ringer's Solution showed no shrinkage and the tissue structure was not affected.

4.4 Discussion

The results showed that all the pathogenic species tested were able to infect the cruciferous and non-cruciferous hosts when artificially inoculated. Though the pathogens were able to infect pepper, tomato and tobacco plants, disease spread was hindered by the elicited hypersensitive response. All the xanthomonads proved to be pathogenic to lettuce by artificial inoculation.

The induction of the hypersensitive response by xanthomonads on pepper, tomato and tobacco plants has also been reported by other researchers (Coutinho & Wallis, 1991; Kearney & Staskawicz, 1990; Sahin & Miller, 1996).

A hypersensitive response is characteristic of a plant resistant to an infecting pathogen in an incompatible plant-pathogen relationship (Kiraly, 1980). Other factors involved in this hypersensitive response include the *hrp* genes and phytoalexins (Kelman, 1979).

Various factors could have contributed to the pathogenicity of the xanthomonads in the test plants, and these include the compatible pathogen-host relationship, the *dsp* and *avr* genes, disruption of metabolism by toxins, tissue degradation and alteration of permeability by proteolytic enzymes, and obstruction of water movement by extracellular polysaccharides (Kiraly, 1980).

The soft rots observed on lettuce plants could be as a result of the action of proteolytic enzymes, phospholipases and bacterial lipomucopolysaccharides (Kelman, 1979). Though soft rots and water-soaking symptoms induced by *X.c. pv. campestris* are not observed in the field on lettuce plants, these symptoms can be experimentally obtained by infiltration of the bacteria in young seedlings or detached host leaves (Kamoun & Kado, 1990).

Artificial inoculation showed the host range of CCS to be similar to those of other xanthomonads. This study confirms the views of other workers (Alvarez *et al.*, 1994; Vauterin *et al.*, 1995) that since some pathovars have overlapping host ranges, a new classification system of *Xanthomonas* species should be created in which both the genomic relationships and the need of plant pathologists for a rational nomenclature are taken into account.

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CHAPTER 5. SCANNING AND TRANSMISSION ELECTRON MICROSCOPE STUDIES OF CRUCIFER CHOCOLATE SPOT

Abstract

Epiphytic and endophytic colonization of cabbage seedlings by the causal agent of crucifer chocolate spot were examined. Seedlings were artificially inoculated, and the resultant infection processes studied by scanning and transmission electron microscopy. Scanning electron microscopy showed cells of the causal organism aggregated around the inoculation sites and stomata. Transmission electron microscopy showed the colonization of xylem vessels, phloem and parenchyma tissues. Profuse multiplication of the pathogen resulted in rupturing of leaf cell organelles and cell membranes.

5.1 Introduction

Phytopathogenic bacteria usually colonize leaf surfaces epiphytically, prior to endophytic colonization and disease development (Rudolph, 1993). Factors that contribute to epiphytic colonization of the host by phytopathogenic bacteria include aerotaxis and chemotaxis of the bacteria toward plant exudates and leachates, motility of bacteria, and electric charges and surface structures of the host and pathogens (Huang, 1986).

Xanthomonas campestris pv. *campestris* (Pammel) Dowson infects the host through hydathodes or wounds (Cook & Robeson, 1986; Dane & Shaw, 1996). This pathogen colonizes the host's vascular system, causing characteristic V-shaped lesions at the leaf margins and vein darkening (Williams, 1980; Bretschneider *et al.*, 1989). The causal organism of crucifer chocolate spot (CCS) closely resembles *X. campestris* pv. *campestris* (Kariem *et al.*, 1995; Laing & Qhobela, 1995). However, its epidemiology and the disease symptoms it causes on different crucifers are different and, therefore, the infection mechanisms of this pathogen may also be different.

Scanning electron microscopy illustrates the relationship of bacteria and surface structures of plant tissues at the sites of infection, whilst transmission electron microscopy reveals ultrastructures of bacteria, plant cells, and bacteria-plant interfaces (Huang, 1986). The distribution of bacterial cells (of the CCS causal agent) in the surface of leaf tissues and in the vascular tissues after artificial inoculation is reported in this chapter.

5.2 Materials and Methods

5.2.1 Inoculation of seedlings

Inoculum of the CCS pathogen (isolate PMK 9608, from Dr Qhobela of the University of Cape Town) was prepared by transferring cells from nutrient agar into nutrient broth. The cells were incubated for 36 h with agitation (150 rpm) at 28°C, and thereafter washed by centrifuging in sterile Ringer's Solution. Cell concentration was adjusted to 10⁸cfu/ml.

Cabbage seedlings cv. Green Star (7-8 weeks old) were inoculated by pricking the leaves with pins and thereafter rubbing in the inoculum with a wad of cotton-wool. Seedlings were kept in a dew chamber at 80-100% relative humidity and 28°C for 48 h. After 48 h, the plants were moved to a greenhouse where the temperature was 22-30°C and relative humidity was 50-60%.

5.2.2 Scanning electron microscopy

Small pieces of tissue (approximately 1.5mm²) were excised from the border region of an infected area and the surrounding healthy tissue and washed with sterile distilled water. This was followed by fixation in 3% buffered glutaraldehyde for eight h. Following fixation, excised tissue was washed in 0.05 M cacodylate buffer (2 x 30 min), and dehydrated in a 30-90% ethanol series for 10 min per concentration. Subsequently, the tissue was transferred to 100% ethanol and dehydrated through a further 2 x 10 min washes. This was followed by drying in a Hitachi Critical Point Dryer HCP-2, and subsequently coating with electron dense Polaron E5100. After coating, specimens were viewed with a Hitachi Scanning Electron Microscope (SEM) S-570. Uninfected tissue (control) was prepared using the same procedure.

5.2.3 Transmission electron microscopy

Excised tissue (as above) was fixed with 3% glutaraldehyde in 0.05 M sodium cacodylate buffer overnight (16 h). This was followed by rinsing in 0.05 M sodium cacodylate buffer for 2 x 30 min, and secondary fixation with 2% osmium tetroxide in 0.05 M sodium cacodylate buffer for 2-4 h. Further rinsing was in 0.05M sodium cacodylate buffer (2 x 30 min washes) and subsequent dehydration was in a series of ethanol (30-100% for 10 min in each solution). Dehydration was completed with two rinses of 15 min each in absolute alcohol. After dehydration, the specimen was washed in propylene oxide (2 x 30 min washes). The specimen was then subsequently placed into three parts propylene oxide: one part Epon-Araldite (2 h); two parts propylene oxide: two parts Epon-Araldite (2 h); and finally, one part propylene oxide: three parts Epon-Araldite (24 h). Following embedding, the specimen was placed in moulds and polymerized for 48 h at 70°C. After polymerization, the specimen was allowed to cool at room temperature (24°C), and sections were then cut with glass knives on a LKB Ultratome III. Sections were stained with 2% uranyl acetate and 2% lead citrate and examined with a Transmission Electron Microscope (TEM) JEOL 100CX.

5.3 Results

5.3.1 Inoculation of seedlings

Inoculation was successful and typical dark, water-soaked spots appeared on the leaves at the pricked regions seven days after inoculation.

5.3.2 Scanning electron microscopy

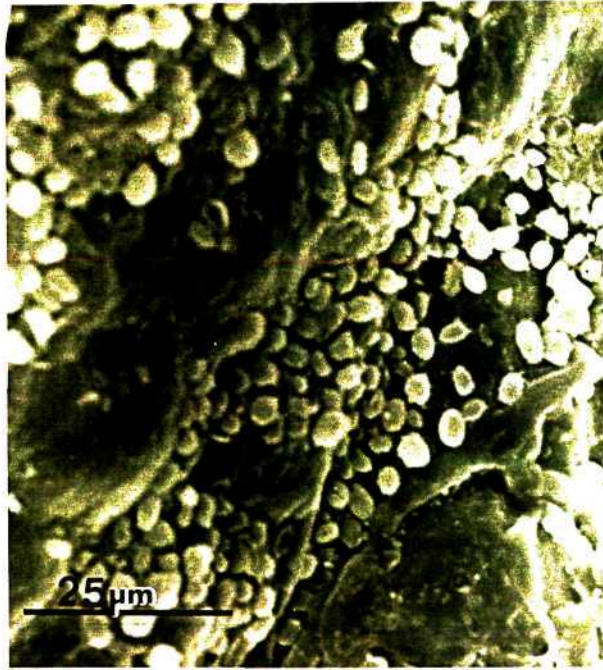


Fig 5.1 Scanning electron micrograph of a cabbage (*Brassica oleracea* var. *capitata*) leaf showing CCS *Xanthomonas campestris* cells (isolate PMK 9608) on the surface of leaf tissue nine days after inoculation

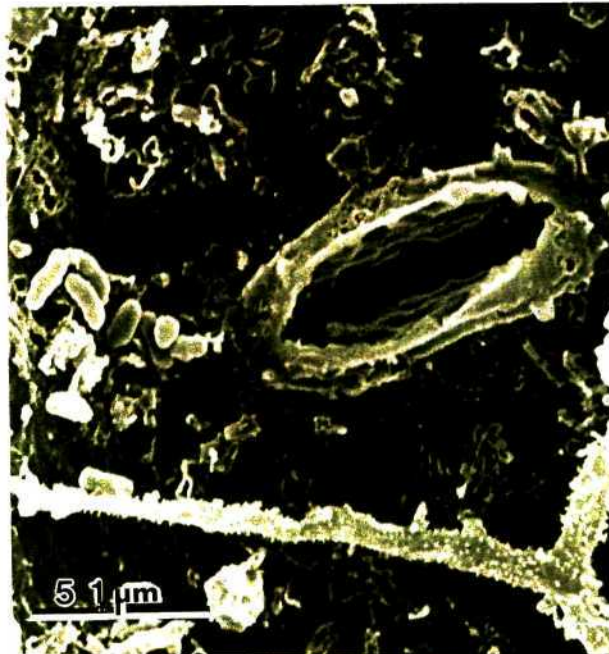


Fig 5.2 Scanning electron micrograph of a cabbage (*Brassica oleracea* var. *capitata*) leaf showing CCS *Xanthomonas campestris* cells (isolate PMK 9608) in the vicinity of a stoma nine days after inoculation

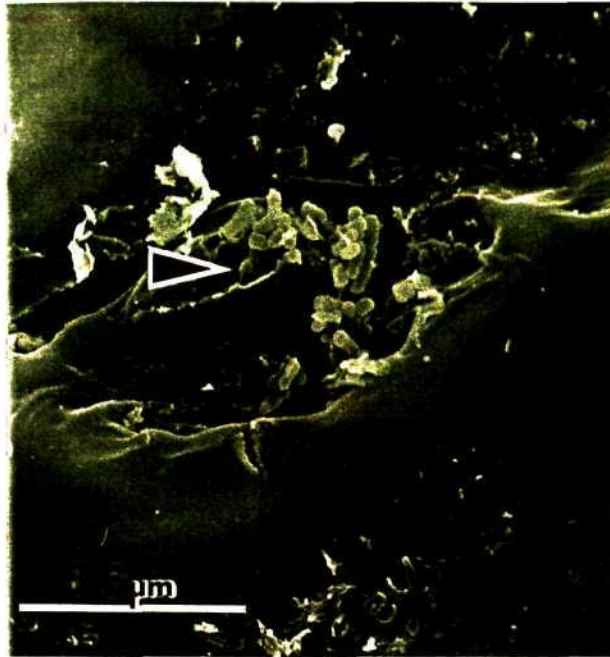


Fig 5.3 Scanning electron micrograph of a cabbage (*Brassica oleracea* var. *capitata*) leaf showing CCS *Xanthomonas campestris* cells (isolate PMK 9608)(arrow) around the inoculation site nine days after inoculation

SEM observations showed epiphytic colonization by xanthomonad cells (Fig 5.1). Colonization was not restricted to points of inoculation and the stomata, but was widespread over the leaf surface. Penetration of the bacteria appeared to be through the stomata and wounds respectively (Fig 5.2 and Fig 5.3). Some rod-shaped bacteria aggregated around these penetration points (Fig 5.3).

5.3.3 Transmission electron microscopy



Fig 5.4 Transmission electron micrograph of a cabbage (*Brassica oleracea* var. *capitata*) leaf showing colonization of host tissues by CCS *Xanthomonas campestris* cells (isolate PMK 9608)(arrow) nine days after inoculation



Fig 5.5 Transmission electron micrograph of a cabbage (*Brassica oleracea* var. *capitata*) leaf showing colonization by CCS *Xanthomonas campestris* (isolate PMK 9608) nine days after inoculation

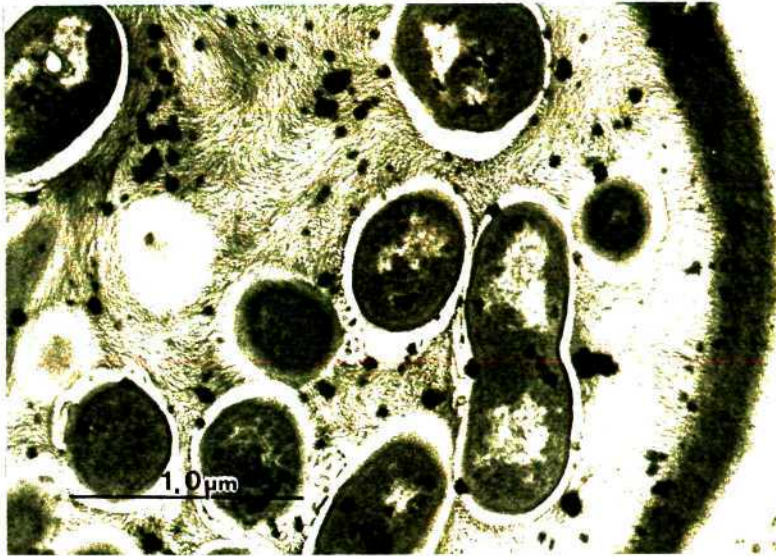


Fig 5.6 Transmission electron micrograph of a cabbage (*Brassica oleracea* var. *capitata*) leaf showing bacterial cells and fibrillar material (arrow) inside the xylem tissues nine days after inoculation

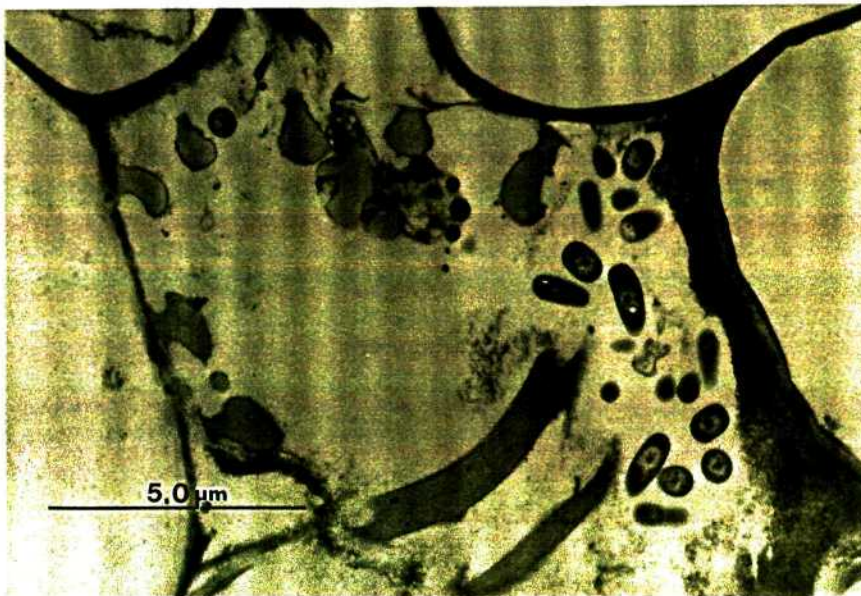


Fig 5.7 Transmission electron micrograph of a cabbage (*Brassica oleracea* var. *capitata*) leaf showing dissolution of host cell walls and tissue collapse, possibly due to bacterial activity nine days after inoculation

The CCS pathogen (isolate PMK 9608) colonized most of the host's tissues, including the spongy parenchyma, phloem and xylem tissues (Fig 5.4). In the initial stages of endophytic colonization, cell organelles remained intact (Fig 5.5). Massive proliferation of the CCS pathogen was accompanied by the release of slime material around the cells, and formation of fibrillar material (Fig 5.6). This also resulted in the plasmolysis of some cell organelles and rupturing of cell membranes (Fig 5.7).

5.4 Discussion

Some tissues of the host that were colonized by the CCS pathogen (isolate PMK 9608) include the vascular bundles, parenchyma tissues and intercellular spaces. Although xylem tissues were also colonized, V-shaped lesions and vein blackening (characteristics typical of *X. campestris* pv. *campestris* infections (Williams, 1980; Shaw & Kado, 1988)) were not observed.

Penetration of the pathogen was through wounds, as SEM observations showed bacterial cells aggregating at the sites of damaged tissue. Previous inoculation studies (refer to Chapter 2) showed that CCS symptoms did not develop without wounding.

Profuse multiplication of the pathogen resulted in the break-down of host cell membranes and dissolution of some cell organelles. This could be due to the action of cellulolytic enzymes, lignolytic enzymes, polygalacturonic acid and transeliminase activity (Wallis *et al.*, 1973). The fibrillar material observed in some invaded host cells might be of plant origin due to bacterial enzyme action on primary walls or it could be bacterial extracellular polysaccharide (Sutton & Williams, 1970; Wallis *et al.*, 1973; Bretschneider *et al.*, 1989).

The pattern of leaf tissue infection by the CCS pathogen confirms the reports of Kariem *et al.* (1995) that the causal agent of CCS is a *Xanthomonas* pathovar. However, the elucidation of the pathovar status of the CCS pathogen rests with a combination of pathogenicity tests, microscopy studies, morphological, biochemical, physiological, serological and molecular biology techniques.

5.5 References

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CHAPTER 6. RESISTANCE AND SUSCEPTIBILITY OF COMMERCIAL CULTIVARS TO CRUCIFER CHOCOLATE SPOT

Abstract

Eighty-six crucifer cultivars from two seed companies were evaluated for their resistance and/or susceptibility to crucifer chocolate spot disease. Cultivars were artificially inoculated using the pin-prick technique. The host response of the various cultivars was variable, with levels of leaf area infected ranging from 0% to 34%. Disease levels were also affected by seasonal variations (summer and winter screening trials), temperature, relative humidity and physiological age of the seedlings.

6.1 Introduction

Crucifer chocolate spot (CCS) is caused by a pathovar of *Xanthomonas campestris* which closely resembles *X.c. pv. campestris* in its physiological and biochemical characteristics. However, the disease differs from black rot in symptomology and epidemiology (Laing & Qhobela, 1995). Although little is known about the resistance and susceptibility of crucifer cultivars to CCS, the following types of defense mechanisms may be expressed to xanthomonad diseases in plants:

1. Passive resistance - this is due to morphological qualities such as altered stomatal structure, fewer hydathodes and post-infection defence structures (Goto, 1992; Kocks & Ruissen, 1996)
2. Active resistance - this is due to actions incited by pathogen attack; e.g., the hypersensitive reaction and synthesis of phytoalexins (Goto, 1992; Kocks & Ruissen 1996)
3. Disease escape - this can be boosted by external factors preventing infection and spread of the disease; e.g., rapidity of germination, hardening of seedlings, spacing of plants, nutrition levels and temperature (Agrios, 1980; Kocks & Ruissen, 1996)
4. Tolerance - this applies to the responses of plants to infection at different levels of biological organization; e.g., cellular tolerance, whole plant tolerance and tolerance through partial escape (Mussel, 1980; Kocks & Ruissen, 1996)

5. Mature plant resistance - this is resistance expressed in mature plants, but not in seedlings (Kocks & Ruissen, 1996).

Artificial inoculation is important during disease screening to counteract avoidance mechanisms (Parlevliet, 1981). During the determination of disease resistance in cultivars, the inoculation method and incubation conditions must be standardized to give reproducible results and produce high levels of disease, but not so severe that plants having some resistance are graded as susceptible (Dhingra & Sinclair, 1985).

Varietal resistance to black rot has been reported by many breeders (Staub & Williams, 1972; Jamwal & Sharma, 1986; Dickson & Hunter, 1987; Tsuji *et al.*, 1991; Msikita *et al.*, 1995). The purpose of this study was to assess the resistance or susceptibility to CCS of a range of crucifer cultivars, made available by two major vegetable seed companies in South Africa.

6.2 Materials and Methods

Inoculum preparation and inoculation

CCS inoculum was prepared by transferring 48 h old cultures (isolate PMK 9608, from Dr. Qhobela of the University of Cape Town) from yeast dextrose carbonate medium into 50 ml nutrient broth in 100 ml Erlenmeyer flasks. The flasks were incubated for 48 h with agitation at 28°C. The inoculum was centrifuged at 3000g and thereafter resuspended in Ringer's Solution. The inoculum density was adjusted to 10⁸cfu/ml.

Inoculation was by pin-pricking the leaves, and thereafter swabbing the injured areas with a cotton wool swab dipped in the inoculum (Sharma *et al.*, 1977). Seedlings were kept in a dew chamber for 48 h after inoculation at a temperature of 28°C and a relative humidity (RH) of 80-100%. After 48 h, the plants were moved to a greenhouse in which the temperature ranged from 16-30°C and the RH of 40-60%.

Screening of genotypes

Thirty cultivars from Starke Ayres and 56 from Mayford Seed Companies were evaluated for their resistance or susceptibility to CCS. The cultivars were provided by the companies as code numbers (without actual names). A popular cabbage cultivar, Hercules lot (AY010R0), was used as a control in two trials, to allow for comparisons between trials run at different times of the year.

Each cultivar was sown in size 24 Speedling® trays, with two trays per cultivar. The seedlings were fertilised with 100 ppm 3.1.3 (38) fertilizer until at 7-8 weeks. The Starke Ayres cultivar trial was conducted during the summer season (January-March 1997) whilst the Mayford cultivar trial was conducted during the winter season (June-August). It was not possible to synchronise the different trials as the seeds were provided at different periods. Disease rating for the percentage leaf area infected was estimated weekly with the aid of a rating scale (Appendix 1). The results were transformed into the area under the disease progress curve (AUDPC) (refer to 3.2.1) and analysed by analysis of variance (ANOVA).

6.3 Results

Table 6.1: Crucifer chocolate spot disease severity on Starke Ayres' crucifer cultivars

Cultivar	Final level of disease severity (%)	AUDPC Values	AUDPC Values as % of Control
Hercules lot AY010RO	21.5	299.75	100
B201	26.5	329	109.8
B202	25.5	312.25	104.2
B203	23	280	93.4
B204	24	300.5	100.3
B205	22.5	265.5	88.6
B206	15.5	213.75	71.3
B207	18	252.37	84.2
B208	19.5	237.75	79.3
B209	22	269.37	89.9
B210	22.5	275.5	91.9
B101	24	314.5	104.9
B102	27	367	122.4
B103	31.5	237.25	79.1
B104	22.5	307.5	102.6
B105	23	299.87	100.0
B106	23	311.75	104.0
B107	24.5	326.5	108.9
B001	22	288	96.1
B002	23.5	311.75	104.0
B003	19.5	249.25	83.2
B004	20	266.37	88.9
B005	22	281.25	93.8
B006	22	302.37	100.9
B007	22	315.5	105.3
B008	24	346.87	115.7
B009	22	297.87	99.4
B010	22.5	327.25	109.2
B011	22.5	299.75	100.0
B012	12.5	202.5	67.6
B013	-	-	-
B014	0	0	0
F Test	4.8		
P level	0.000		
CV%	5.0		
LSD(0.05)	68.5		

Table 6.2: Crucifer chocolate spot disease severity on Mayford's crucifer cultivars

Cultivars	Final level of disease severity (%)	AUDPC Values	AUDPC Values as % of control
Hercules lot AY010R0	14.75	273	100
B016	14.25	281	102.9
B017	15.25	268	98.2
B018	15.25	286	104.8
B019	14	266	97.4
B020	15.75	306	112.1
B021	15.5	302	110.6
B022	16	307	112.5
B023	16.5	299	109.5
B024	15	276	101.1
B025	0	0	0
B026	15	285	104.4
B027	15	284	104.0
B028	15	279	102.2
B029	16.5	302	110.6
B030	14.25	286	104.8
B031	-	-	-
B032	14.25	291	106.6
B033	15.5	304	111.4
B034	14.75	295	108.1
B035	14	288	105.5
B036	15.75	293	107.3
B037	14.25	293	107.3
B038	14.25	292	107.0
B039	15	281	102.9
B108	18.5	353	129.3
B109	16.75	296	108.4
B110	16.75	295	108.1
B111	17.75	319	116.8
B112	16.75	316	115.8
B113	17.25	313	114.7
B114	19	340	124.5
B115	16.5	315	115.4
B211	17.5	330	120.9
B212	15.75	294	107.7

B213	18	335	122.7
B214	21.25	367	134.4
B215	17.5	319	116.8
B216	18	340	124.5
B217	16.25	320	117.2
B301	15	293	107.3
B302	-	-	-
B401	7	100	36.6
B402	7.5	140	51.3
B403	7.5	130	47.6
B404	5.25	127	46.5
B405	6.25	106	38.8
B501	6.5	109	39.9
B502	6	120	44.0
B601	7	123	45.1
B602	5.5	128	46.9
B603	6.75	105	38.5
B604	5.5	130	47.6
B605	6.5	119	43.6
B606	4.25	124	45.4
B607	5.5	67	24.5
B608	14.25	92	33.7
F test	79.3		
P Level	0.000		
CV%	9.77		
LSD (0.05)	29.1		

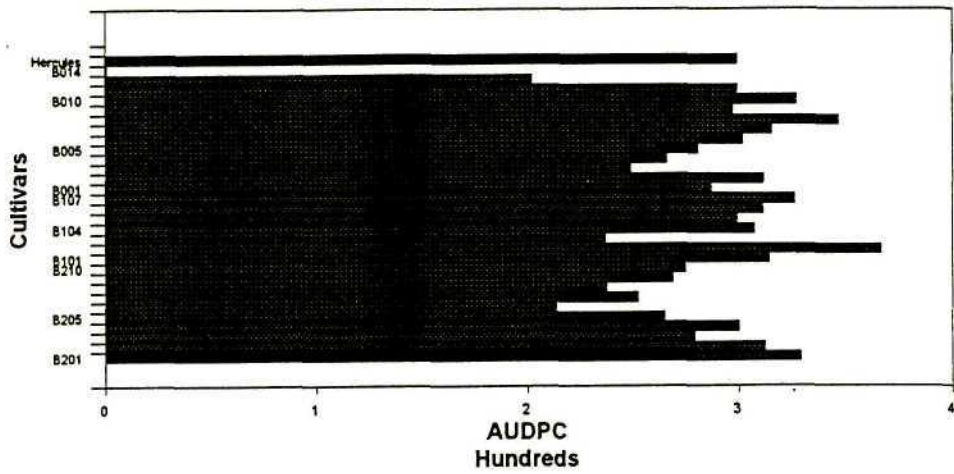


Fig 6.1 The responses of various Starke Ayres' crucifer cultivars to crucifer chocolate spot disease (caused by isolate PMK 9608)

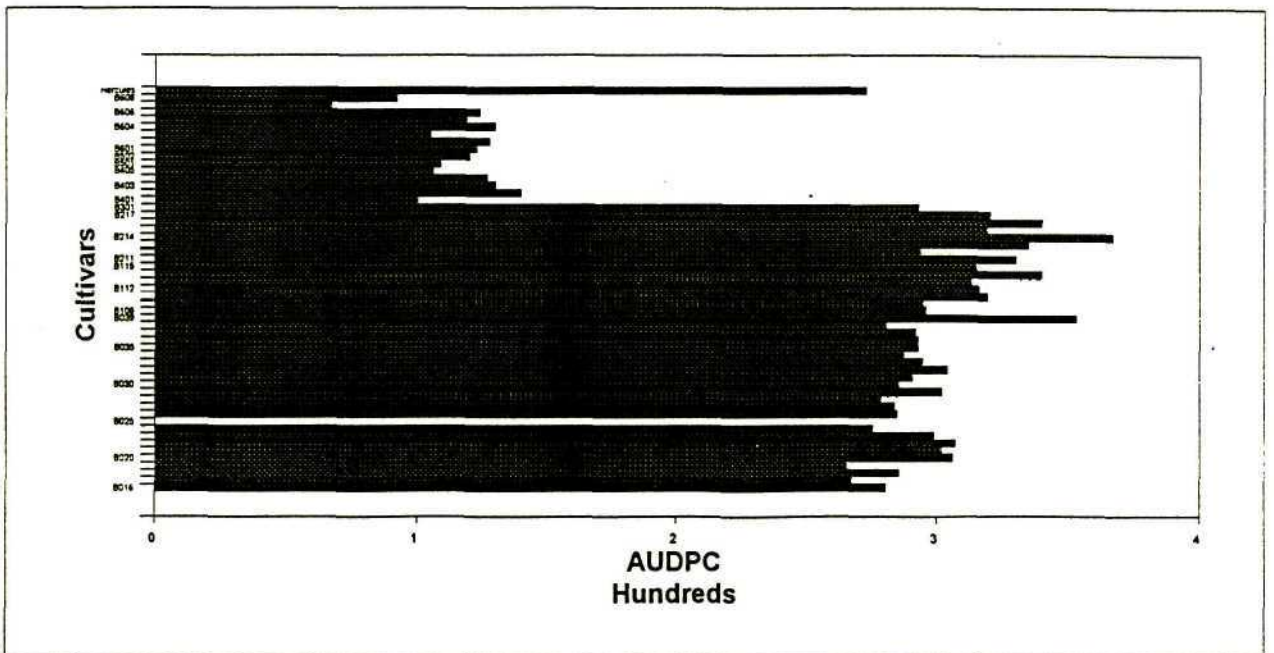


Fig 6.2 The responses of various Mayford's crucifer cultivars to crucifer chocolate spot disease (caused by isolate PMK 9608)

There was considerable variation in the susceptibility or resistance of various cultivars to CCS (Fig 6.1 and Fig 6.2). No infection could be obtained when cultivars B025 or B014 were inoculated with the CCS organism (Fig 6.1 and Fig 6.2, respectively).

When cultivars B401, B402, B403, B404, B405, B501, B502, B601, B602, B603, B604, B605, B606, B607 (Fig 6.1) and B012 (Fig.6.2) were inoculated, the symptoms of CCS disease did not develop significantly. The highest level of disease was obtained when cultivars B102, B107 B108, B114, B201, B213, B214 and B216 were infected with the CCS organism. Two cultivars, B013 and B031 had a low germination percentage, thus the disease levels were not truly representative of the particular treatments (Tables 6.1 and 6.2).

6.4 Discussion

The responses of crucifer cultivars to the CCS pathogen (PMK 9608) were variable. Although the disease did not develop in B014 and B025, the two cultivars cannot be considered immune as resistance is relative (Milus *et al.*, 1996). The distinction between compatibility and incompatibility is one of pathogen/host specificity (Cook & Robeson, 1986). The interaction of resistance genes and avirulent genes (*avr*) might have resulted in the elicitation of a hypersensitive response in cultivars B401-B608.

The use of a high inoculum dose (10^8 cfu/ml) ensured the expression of distinct differences in the resistance/susceptibility of cultivars to CCS disease; compensated for the less than optimal conditions; and reduced the time required for symptom development (Dhingra & Sinclair, 1985). Such differences cannot be revealed between cultivars when the inoculum dose is low (Staub & Williams, 1972; Goto, 1992). Other factors that influenced the production of symptoms are temperature, relative humidity, light, age of seedlings, the inoculation method and genotypes used (Sharma *et al.*, 1977; Dane & Shaw, 1993; Msikita *et al.*, 1995; Kocks & Ruissen, 1996; Milus, 1996).

The results of this investigation indicate that crucifer seedlings vary in their levels of susceptibility/resistance to Chocolate spot. They also show that some cultivars could not be infected under the experimental conditions employed. This suggests that the possibility exists for breeding resistant genotypes as a management option for control of CCS.

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CHAPTER 7. TESTING OF BRASSICA SEED FOR PRESENCE OF THE CRUCIFER CHOCOLATE SPOT PATHOGEN AND TREATMENT OF CONTAMINATED SEED

Abstract

Methods of seed testing for the detection of the causal agent of crucifer chocolate spot, and seed treatment were investigated. Recovery of the pathogen (isolate PMK 9608) was most successful on nutrient starch cycloheximide antibiotic agar (NSCAA) medium. Seed treatment with biocides was more effective than hot water treatment in lowering seedborne pathogen (isolate PMK 9608) levels. However, percentage germination of seeds was low after chemical treatment, while hot water treatment was less damaging. All seed treatments lowered pathogen levels, but none resulted in complete elimination of seedborne inoculum.

7.1 Introduction

Crucifer chocolate spot (CCS) is caused by a xanthomonad pathogen closely related to *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson which causes black rot disease of brassicas, and for which contaminated seed is considered the main source of infection (Minchinton, 1996).

Several laboratory assays have been developed to detect *X. campestris* pv. *campestris* in contaminated seed:

- i) Germination of seed *in vitro* or *in vivo* and observing seedlings for symptoms such as yellowing, blackening and progressive collapse (Shackleton, 1962; Franken *et al.*, 1991).
- ii) Observation of dry seeds under a microscope or UV light for impurities such as debris, and also for symptoms such as discolouration, malformation, mechanical damage and presence of bacterial masses (Neergaard, 1977; Agarwal & Sinclair, 1987a)
- iii) Plating of seeds onto semiselective media (Chang *et al.*, 1991)

- iv) Staining of liquid smears of seed extracts using immunofluorescence (Schaad & Donaldson, 1980)
- iv) Plating of liquid samples of seed extracts on agar media after washing and soaking the seeds (Schaad & Donaldson, 1980)

Following isolation from contaminated seed, the organism has to be identified. Techniques used in the confirmation of xanthomonad isolates include:

- i) Gram-stain, study of cell and culture morphology (Schaad & Stall, 1988)
- ii) Physiological and biochemical techniques; e.g., growth on semiselective media, utilization of sugars and other oxidizable substrates (Dye, 1962; Chun & Alvarez, 1983; Franken *et al.*, 1991)
- iii) Serological techniques; e.g., the Immunofluorescence (IF) assay, Enzyme-Linked-Immunosorbent-Assay (ELISA) using monoclonal and polyclonal antibodies, etc.; sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of membrane proteins, lipopolysaccharides, etc.; restriction fragment length polymorphism (RFLP) patterns of genomic DNA (Schaad, 1982, Franken, 1992a,b).
- iv) Pathogenicity tests; e.g., spraying purified suspensions of the isolated bacterium with a chromatographic sprayer onto leaves (Schaad³, 1996: pers. comm.).

Seeds may be treated by biological, chemical, or physical processes in order to control seedborne organisms (Agarwal & Sinclair, 1987b).

There is not much evidence for the control of *Xanthomonas* sp. on seed by antagonists, although Maude (1996) reported that strains of *Erwinia herbicola* (Lohnis) Dye, applied in suspension to cotton seeds naturally infected with *X. campestris* pv. *malvacearum* (Smith) Dye, considerably reduced black arm infection in the emerged seedlings.

³Schaad, N.W. 1996. Department of Plant Pathology, University of Georgia, USA.

Chemical treatment relies on the use of various type of chemicals (e.g., calcium hypochlorite, sodium hypochlorite, streptomycin) in different formulations (e.g. wettable powders, dusts, slurries or suspensions) (Neergaard, 1977; McKeen, 1981; Agarwal & Sinclair, 1987b). However, antibiotics are not widely used due to the potential hazard of transfer of drug resistance to plant pathogenic species (Mew & Natural, 1993).

The principle of physical treatment or thermotherapy is that microorganism are killed at temperatures that are tolerated by seeds (Agarwal & Sinclair, 1987b). Methods applicable in this technique include treatment by hot water, aerated steam, dry heat, solar heat and electromagnetic radiation (Maude, 1996). The objectives of this study were to test for the presence of the CCS causal agent in seed, and to assess the effect of some physical and chemical treatment techniques on the viability of the CCS causal organism.

7.2 Materials and Methods

The seed lots used in this study were obtained from various seedling growers in South Africa from 1990-1995. Two methods were used for testing. The first method entailed direct plating of seed on semi-selective media (Schaad, 1982). The second method involved plating of seed extracts (Schaad, 1989).

7.2.1 Direct plating of seed onto semi-selective media

The seed was soaked in 1:10 diluted 5.25% sodium hypochlorite for three min, followed by three rinses in sterile distilled water. The seed was then left to dry on a laminar flow bench, and thereafter plated onto semi-selective media. The semi-selective media used were starch-*Xanthomonas* (SX) medium (Schaad & White, 1974a), *Xanthomonas campestris* pv. *pruni* selective (XPS) medium (Civerolo *et al.*, 1982), nutrient starch cycloheximide antibiotic agar (NSCAA)(Randhawa & Schaad, 1984) and FS medium (Yuen *et al.*, 1987).

Six replicates of twenty seeds from each seedlot were distributed on each of the above agar media. The seed lots tested by this technique are shown on Table 7.1.

Table 7.1 Crucifer seed lots tested by direct plating of seed onto semi-selective media

Cultivar	Lot No
Prince Marvel	940122
Incline	940260
Star 6601	20990I
Wallaby	HT302049
Hunter	304D4SIQ
Glacier	920110
Snowcap	OBW302V
Hunter	HT301132
Spring Snow	930303
White Rock	0376
Rami	8H836
Green Valiant	940214
Star 4402	-
Glacier	911151
Green Coronet	138294941y
Tenacity	11832
Green Coronet	1392 T4941y
Green Crown	-
Conquistador	12378
Conquistador	12492
Markanta	FGKB10A0370
Star 3301	-
Green Crown	-

7.2.2 Soaking of seeds and plating of liquid extracts onto semi-selective media

About 40g of seed per sample (Table 7.2) were added to 100ml Tween 20-saline solution. These were agitated for two h at 4⁰C, and then filtered through sterile cheese cloth into centrifuge tubes. The samples were centrifuged at 10000g for 10 min, the tubes drained, the resultant pellet resuspended in Tween-saline solution, and serially diluted (10⁻¹-10⁻²). Aliquots (0.1) ml were pipetted from the 0, 10⁻¹ and 10⁻² dilutions (in triplicate) onto basal starch cycloheximide antibiotic agar (Randhawa & Schaad, 1984), NSCAA (Randhawa & Schaad, 1984) and FS (Yuen *et al.*, 1987) selective media. The plates were incubated at 30⁰C for 2-3 days. Testing of most seed lots (Tables 7.1 and 7.2) could not be done by both methods due to the limited number of seeds available.

Table 7.2 Crucifer seed lots tested for the CCS pathogen by plating of seed washings

Name	Lot No
Prominence	S00777/A
Topmost	E2373A/3402
Premium Crop	R0I022
Green Star	9172941A29
Snowcap	OBW302V
Kompactica	SIN:11280
Hercules	910762
Markanta	-
Wallaby	-
Hercules	33127
Grandslam	12578
Caliber	920132
Green Star	X01503H
Green Star	917294A29
Conquistador	12378
Shantung	-
Small Prince	HT310088
Prominence	-
Bonanza	-

7.2.3 Identification and characterization of xanthomonad colonies

Suspect *Xanthomonas* colonies were transferred to plates of yeast extract-dextrose-CaCO₃ (YDC) medium (Schaad, 1989). Characterization of the isolates was achieved through Gram staining, observing colony morphology on YDC, acid production from glucose and mannose, oxygen requirements and starch hydrolysis (Schaad, 1989).

Isolates provisionally identified as the CCS pathogen were sent to the University of Cape Town (UCT) for positive identification by the use of sodium-dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and studying restriction fragment length polymorphism (RFLP) patterns of genomic DNA.

7.2.4 Pathogenicity tests

Presumptive isolates of the CCS pathogen were inoculated into nutrient broth and incubated on an orbital shaker for 24 h at 28°C, followed by centrifugation at 10000g for 10 min. The supernatant was discarded and the pellet suspended in sterile Ringer's Solution. The inoculum concentration was adjusted to 10⁸ cfu/ml. Eight week-old cabbage seedlings cv. Perfection Cross and Baby Cabbage (in speedling® 24 trays) were then inoculated (four replicates) by pin-pricking their leaves, and wiping the wounds with a cotton-wool swab dabbed in the centrifuged inoculum. The inoculated plants were kept in a dew chamber at 28°C and relative humidity (RH) of 80-100% for 48 h. Thereafter, the plants were moved to a greenhouse (RH 50-60%, temperature 22-30°C) and observed for symptoms over a four week period.

7.2.5 Seed treatment and its effect on germination percentage

In each treatment, about 10000 cauliflower seeds (lot OBW302V) were artificially infected with the CCS pathogen (isolate PMK 9608) by soaking in a 10⁸cfu/ml bacterial suspension for 24 h. Isolate PMK 9608 was isolated and characterized at UCT (Qhobela⁴, 1996: pers. comm.). The seeds were then left to dry on a laminar flow bench, and thereafter subjected to various treatments (Table 7.3).

⁴Qhobela, M., Department of Microbiology, University of Cape Town, Rondebosch, 7700.

Table 7.3 Treatments applied to artificially contaminated *Brassica* seeds

Treatment	Method
Hot water	Soaking seed in hot water at 50°C for 30 min (Clayton, 1924; Agarwal & Sinclair, 1987a)
Hot water and 1:10 NaOCl	Soaking seed in 1:10 NaOCl at 50°C for 30 min (Babadoost <i>et al.</i> , 1996)
Kocide 101	Seed-soaking in 3g/l of Kocide 101 at 30°C for two h
Copper ammonium carbonate B (CAC B)	Seed-soaking in 5g/l of CAC B at 30°C for two h
Copper oxychloride	Seed-soaking in 4g/l of CuOCl at 30°C for two h
Inoculated control	Seed treated with the inoculum only
Uninoculated control	Untreated control (treated with neither chemicals nor inoculum)

All the treatments were performed in a shaker-waterbath. After treatment, the seeds were rinsed twice with sterile distilled water and left to dry on a laminar flow bench. Liquid extracts made from these seeds were plated (as in 7.2.2) on NSCAA and BSCAA.

Percentage seed germination was assessed after the various treatments (25 seeds/plate, four replicates) by placing seeds in 90mm petri dishes layered with blotting paper. The blotting paper was moistened with distilled water daily. The results were analysed by multifactor ANOVA.

7.3 Results

7.3.1 Direct plating of seed on semi-selective media

Typical xanthomonad colonies were detected from Prince Marvel 940122, Rami 8H836 and Markanta FGKB10A0370. Colonies developed sooner on NSCAA and XPS media, than on FS and SX media.

7.3.2 Soaking of seed and plating of liquid samples onto semi-selective media

Xanthomonad-like colonies were detected from Kompactica SIN:11280, Hercules 910762, Wallaby, Hercules 33172, Caliber 920132, Green Star 917294A29, Conquistador 12378, Small Prince HT310088, Prominence and Bonanza. Colonies appeared sooner on NSCAA than on the other semi-selective media tested.

7.3.3 Identification and characterization of xanthomonad colonies

The bacteria (from 7.3.1 and 7.3.2) were typical xanthomonads showing the characteristics of being aerobic, Gram negative, mucoid, yellow in colour, starch hydrolysing, and produced acid from glucose and mannose. However, characterization by serological techniques (at UCT) showed that none of the isolates were related to the CCS pathogen.

7.3.4 Pathogenicity tests

Symptoms of systemic yellowing and blackening around the points of inoculation were observed only when cultures from the following seed lots were inoculated into test seedlings: Prince Marvel 940122, Rami 8H836, Markanta FGKB10A0370, Wallaby, Green Star 917294A29, Conquistador 12378, Prominence and Bonanza.

7.3.5 Seed treatment and its effect on percentage germination

The recovery of the CCS pathogen (isolate PMK 9608) was low after Kocide 101 treatment, and high after hot water treatment (Table 7.4). The growth of the CCS pathogen (isolate PMK 9608) was faster on NSCAA than on BSCAA. The germination percentage was high after hot water treatment, and low after Kocide 101 treatment.

Table 7.4 Effect of seed treatment on the crucifer chocolate spot pathogen (isolate PMK 9608) and on percentage seed germination

Seed Treatment	Mean No of Colonies on NSCAA	Mean No of colonies on BSCAA	Germination of seed (Mean %)
Hot water (50°C 30 min)	46.5 c	30.5 b	54 c
1:10 NaOCl (50°C 30 min)	38.5 b	27.5 b	45 b
3g/l Kocide (30°C 2 hr)	28 a	16.5 b	35 a
5g/l CAC B (30°C 2 hr)	32.5 b	19 b	44 b
4g/l CuOCl (30°C 2 hr)	31.5 b	26.8 b	42 b
Inoculated control	>300 d	132.3 a	66 d
Uninoculated control	0 d	0 b	72 d
F Test	2897.9	13.8	38.0
P Level	0.000	0.000	0.000
CV%	3.8	51.3	5.0
LSD (0.05)	5.7	47.3	6.5

Values with the same letter do not differ significantly at the level, $P = 0.05$, using Fisher's LSD Test.

7.4 Discussion

The CCS pathogen was not detected in any of the seed lots tested in this study. However, a negative result does not guarantee the seed lot is disease-free, only that in the sample tested by that method, the pathogen was not detected. Although the CCS pathogen was not detected in this study, contaminated seed is known to be a source of primary inoculum for xanthomonad diseases (Schaad & White, 1974b; Lunsgaard, 1976; Schaad, 1989; Franken *et al.*, 1991; Babadoost *et al.*, 1996).

As xanthomonad-like isolates from some seed lots did not evoke symptoms after artificial inoculation of brassica seedlings, they might have been saprophytic xanthomonads or pathogens that have lost their virulence (Cook *et al.*, 1952; Chang *et al.*, 1991). Randhawa & Schaad (1984) also found that not all colonies positively identified as *X. campestris* pv. *campestris* were pathogenic to brassica seedlings. As previously reported by Chang *et al.* (1991), the addition of antibiotics and the application of surface sterilization did not completely inhibit the saprophytes associated with the seed in this study.

All seed treatments significantly reduced the recovery of the CCS pathogen (isolate PMK 9608) from artificially inoculated seed. Chemical treatment of seed was found to be more effective than hot water treatment. Several authors have found that hot water treatment was not effective in controlling xanthomonad diseases as numbers of bacteria recovered from the tested seed could still be high enough to lead to an epidemic in the field (Humaydan *et al.*, 1980; Schaad, 1982). Although Babadoost *et al.* (1996) found seed treatment by NaOCl to be highly effective, it was not more effective than copper treatments in the present study.

Percentage germination of seed was low after seed treatment by all the tested biocides, compared to hot water treatment. This was possibly due to phytotoxic effects of copper chemicals (Humaydan *et al.*, 1980; Schultz *et al.*, 1986). Overall percentage germination in this trial was low; probably due to the old seed lot used (Snowcap OBW302V) as its vigour may have deteriorated with time. This old seed lot (Snowcap OBW302V) was used as it was the only one available in bulk (>300 g) for utilization in this research project. Age and condition of seeds will also affect their response to seed treatments (Minchinton, 1996).

Therefore, it is important for growers to select from various types of seed treatment methods depending on the condition of the seeds to be treated. As seed treatment does not result in complete eradication of seedborne pathogens (Minchinton, 1996), it is important to integrate it with other disease control programmes in the management of CCS disease.

Future research needs to concentrate on the effect of seed treatment on percentage germination of seed of different ages and of different cultivars. A wider range of biocides should also be tested, especially modern quaternary ammonium compound formulations.

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CHAPTER 8. THE EFFECT OF NUTRITION ON THE SEVERITY OF CRUCIFER CHOCOLATE SPOT IN CABBAGE SEEDLINGS

Abstract

Cabbage (*Brassica oleracea* var. *capitata*) seedlings (cv. Green Star) were grown with different levels of 3.1.3 (38) water-soluble fertilizer prior to inoculation with the pathogen of crucifer chocolate spot (CCS) disease. Seedlings were inoculated when 7-8 weeks old, and the severity of CCS assessed five days after inoculation. Disease levels were reduced at higher levels of fertilizer application. Elevated amounts of fertilizer resulted in increased levels of N, P, K, Zn and Cu, whilst Mg, Na, and Ca remain unaffected in the leaves of cabbage seedlings.

8.1 Introduction

Increases in the costs of agrochemicals and resistance of some plant pathogens to pesticides has made the use of cultural methods, such as the manipulation of plant nutrition, important options in the commercial production of crops. The nutrition of a plant may determine its resistance or susceptibility to disease, its histological or morphological structure or properties, the function of tissues to hasten or retard pathogenesis, and the virulence and ability of pathogens to survive (Huber, 1974; Huber, 1980). This is, however, a contentious issue (Nowell, 1997). Plant nutrition affects disease severity by increasing physiological tolerance through compensation for pathogenic damage and enhancing physiological resistance of the plant (Huber, 1978; Huber, 1980; Goto, 1992).

Some pathogens prefer to attack stressed plants, and are called low sugar diseases, whereas others attack lush plants and are called high sugar diseases (Horsfall & Diamond, 1957). Most low sugar disease processes are biotrophic, whereas most high sugar disease processes are necrotrophic (Vanderplank, 1984).

There are contradictory reports in the literature concerning the effect of nutrient levels on *Xanthomonas* diseases. Application of high levels of nitrogen have been associated with high levels of bacterial blight of rice (*Oryzae sativa* L.) caused by *X. campestris* pv. *oryzae* (Ishiyama) Swings *et al.*, bacterial leaf streak of rice caused by *X.c.* pv. *oryziola* (Fang *et al.*) Swings *et al.* and leaf blight of clusterbean (*Phaseolus vulgaris* L.) caused by *X.c.* pv. *cyamopsidis* (Patel *et al.*) Dye (Ho & Lim, 1979; Choi *et al.*, 1980; Pandey & Iswaran, 1982; Gandhi & Chand, 1985; Reddy *et al.*, 1989). Application of microbial fertilizers (in the form of *Azotobacter* and *Pseudomonas* spp.) on plants infected with bacterial blight aggravated the "kersek" and "leaf blight" phases of the disease (Pandey & Iswaran, 1982).

Paradoxically, increased application on nitrogen (either in combination with or unaccompanied by potassium and phosphorus) has been implicated in the decreased severity of leaf blight of *Synogonium podophyllum* "White Butterfly" caused by *X.c.* pv. *synogonii* (Dickey & Zumoff), bacterial leaf spot of tomatoes (*Lycopersicon esculentum* L.) caused by *X.c.* pv. *vesicatoria* (Doidge) Dye, and bacterial blight of philodendron (*Philodendron selloum* L.) caused by *E. chrysanthemi* pv. *philodendron* (Burkholder *et al.*) (Nayudu & Walker, 1960; Chase & Poole, 1987; Chase, 1989; Chase, 1990; McGuire *et al.*, 1991; Goto, 1992).

In South African nurseries, the Speedling® system is used extensively, usually in conjunction with a hydroponic fertilizer. Thus, the fertilizer level applied is controlled by the grower, and balance of nutrients is essential for the optimum growth of plants. This experiment was set out to assess the effect that increased levels of nutrients have on CCS.

8.2 Materials and Methods

Seedlings

Two to three week old cabbage (*Brassica oleracea* var. *capitata*) seedlings cv. Green Star 340301/07030 grown on pine bark medium were supplied by a nursery in size 200 Speedling® trays. The seedlings were transferred to size 24 Speedling® trays and Ocean 3.1.3 (38) hydroponic fertilizer applied (three times a week) at 4 g/l, 2 g/l, 1.5 g/l, 1 g/l, 0.5 g/l and 0 g/l (control). The nutrients present in this fertilizer were 162 g nitrogen, 55 g phosphorus, 162 g potassium, 6 g magnesium and 50 g sulphur per Kg product.

Inoculum preparations

A loopfull of growth from 36-48 h old cultures of CCS (isolate PMK 9608, from Dr Qhobela of the University of Cape Town) on nutrient agar was transferred to 75 ml nutrient broth in 250 ml Erlenmeyer flasks. The flasks were shaker-incubated (150 rpm) at 30°C for 36-48 h, after which the cells were washed by centrifuging in a J2-HS Beckman centrifuge at 3000g for 10 mins, and thereafter resuspended in sterile Ringer's solution to give a final concentration of 10⁸cfu/ml.

Inoculation of 7-8 week old seedlings was done by pricking the leaves with pins and then rubbing in the inoculum by using a wad of cotton wool. The plants were moved to a dew chamber at 80-100% relative humidity and 28-30°C for 48-72 h. The inoculum was also applied by spraying with an atomizer immediately after wounding and again, 24 h later. The inoculated seedlings were then left in the dew chamber for 48 h (12 h day-night photoperiods). The plants were moved to a glasshouse where the temperature was 27-30°C and the relative humidity was 50-60%. Disease ratings commenced five days after inoculation. The results of this experiment were analysed by ANOVA of the AUDPC (area under the disease progress curve) (refer to 3.2.1) using Statsgraphics.

Leaf analysis

After the final disease rating, leaf samples from various treatments were sent to the Feed/Plant Laboratory at Cedara College to be analysed for their protein, macro- and micronutrient content.

8.3 Results

Table 8.1 Effect of 3.1.3 (38) fertilizer treatments on chemical composition of cabbage leaf tissue

DATA ON 100% DRY MATTER BASIS										
Fertilizer* level (ppm)	Protein content of leaf tissue	N	Ca	Mg	K	Na	P	Zn	Cu	Mn
400	38.07	6.09	0.55	0.33	3.26	0.30	0.83	64	27	109
200	21.94	3.51	0.62	0.41	2.95	0.32	0.52	27	14	63
150	18.96	3.03	0.41	0.33	2.51	0.27	0.53	33	12	47
100	12.19	1.95	0.58	0.37	2.17	0.27	0.41	27	12	59
50	13.35	2.14	0.63	0.42	2.34	0.30	0.42	29	52	66
0	11.26	1.80	0.55	0.37	2.00	0.38	0.45	26	22	53

*Ocean 3.1.3 (38) fertilizer

Table 8.2 Effect of 3.1.3 (38) fertilizer levels on crucifer chocolate spot disease severity

Fertilizer* level (ppm)	Final disease rating as % leaf area infected	AUDPC	AUDPC as % of Control
400	15.3	235.7 d	52.2
200	18.2	283.8 c	62.8
150	23.5	376.7 b	83.3
100	24.4	392.9 b	86.9
50	25.2	397.3 b	87.9
0	28.8	452 a	100
F Test	36.1		
P Level	0.000		
CV%	41.6		
LSD (0.05)	37.4		

Values with the same letter do not differ significantly at the level, $P = 0.05$, using Fisher's LSD Test.

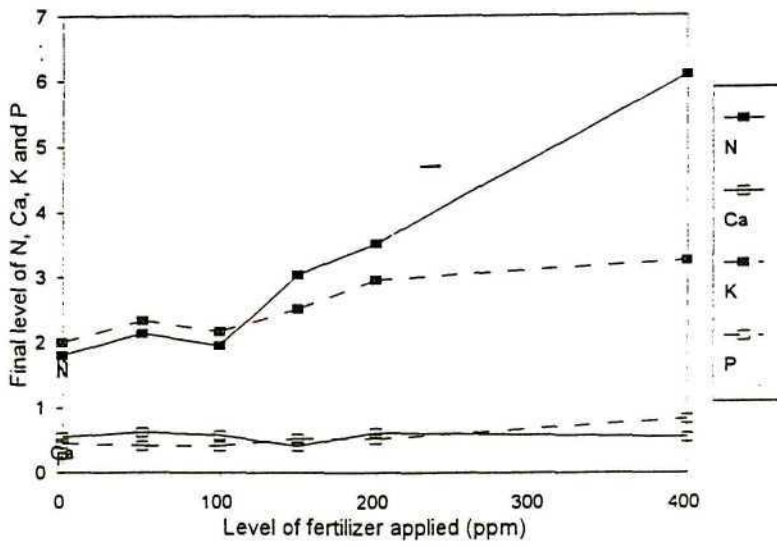


Fig 8.1 Chemical composition of cabbage leaf tissue after different levels of fertilizer application

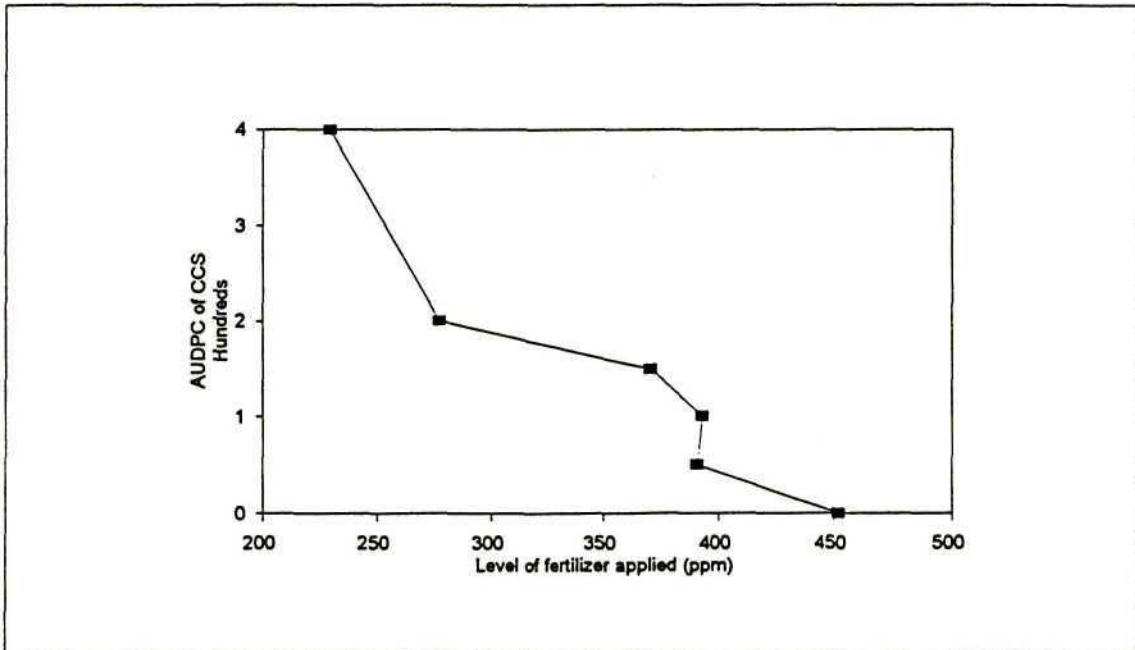


Fig 8.2 The effect of fertilizer levels on crucifer chocolate spot disease severity

Symptoms were most severe when no fertilizer was applied and least severe at 400 ppm Ocean 3.1.3 (38) (Fig 8.2). Leaf analysis showed a linear relationship between the chemical composition of leaf tissue and the concentration of fertilizer applied (Table 8.1 and Fig 8.1). With the exceptions of magnesium, sodium and calcium, seedlings that received 400 ppm fertilizer had higher content of protein, nitrogen, phosphorus and potassium than those to which lower levels of fertilizer were applied (0 - 200 ppm). The levels of magnesium, sodium and calcium were not affected by the fertilizer levels applied. Levels of zinc and copper varied between 50 and 200 ppm, with the control leaves showing the lowest levels and the most heavily fertilized plants the highest levels.

8.4 Discussion

The severity of CCS (isolate PMK 9608) was lower on plants that received high levels of 3.1.3 (38) fertilizer. This result is similar to those reported for some other xanthomonad diseases (Harkness & Marlatt, 1970; Chase, 1989; Goto, 1992). Nutrients that increased in the leaf tissue with fertilizer applications included N, P, K, Mn and Cu, whereas, other nutrients like Ca, Mg, Na and Mg showed an inconsistent variation. This confirms literature reports (Huber, 1980; Gvodyzak *et al.*, 1986) that N, P, K, Mn and Cu are essential in building-up defence responses to xanthomonad pathogens. However, this contradicts other reports (Choi *et al.*, 1980; Devadath *et al.*, 1987; Rudolf, 1993) that showed that it is only increased levels of calcium and potassium that result in reduced severity of *Xanthomonas campestris* elicited symptoms. While discussing the elements individually, it must be borne in mind that it is an interaction of elements that determines the nutrient balance of plants, and that there are differences between genotypes in their ability to take up the various nutrients (Farina *et al.*, 1983; Nowell, 1997).

The sum of many interacting factors of the pathogen, host, environment, chemical constituents of the fertilizer applied, enhanced production of enzymes and antibacterial compounds, and time, determine how a disease is affected by the host plants' nutritional status (Huber, 1980; Phillip & Devadath, 1984).

For plants that respond adversely to a two- or three-fold increase in fertilizer level (over the optimum level for plant growth), it is unlikely that any reduction in disease severity would result from applying more than this optimum amount for bacterial disease reduction (Chase, 1989).

The level of fertilizer applied to crucifer seedlings in South African nurseries ranges from zero to 1000 ppm depending in the stage of the production stage (refer to Chapter 10). Thus, CCS becomes highly prevalent on seedlings in the hardening-off cycle of production. The chemical composition of the seedlings at different ages also influences the nutritional status of the plants, and thus vulnerability to the disease (Laing, 1996).

The results of this trial indicate that CCS can be managed by avoiding nutrient stress of the seedlings. Under nursery conditions, biotic and abiotic factors must be correlated with the fertilizer programmes to minimize costs.

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CHAPTER 9. CHEMICAL CONTROL OF CRUCIFER CHOCOLATE SPOT

Abstract

Several bactericides were assessed for their efficacy in controlling crucifer chocolate spot and for their phytotoxicity to crucifer seedlings. Disease reduction ranged from 0-32%. Copper compounds were more effective than quaternary ammonium compounds in reducing disease levels. The performance of the copper bactericides was improved when these compounds were combined with mancozeb. Application of copper bactericides up to four times the recommended dosage did not result in phytotoxic effects.

9.1 Introduction

Chemical control aims at protecting the plants from pathogen inoculum that is likely to arrive, and at curing an infection that is already in progress (Agrios, 1988). Chemicals used in the control of bacterial plant diseases include antibiotics, fungicide/bactericide mixtures and bleaching agents such as sodium hypochlorite or calcium hypochlorite (Agrios, 1988; Natrajan *et al.*, 1988; Heitefuss, 1989).

There are only a few bactericides among the large number of antimicrobial compounds manufactured for plant disease control (Mew & Natural, 1993). This is due to the emphasis on control of fungal plant diseases compared to that of bacterial plant diseases and the concern caused by rapid development of resistant strains in phytobacterial populations (Jones *et al.*, 1991; Sundin & Bender, 1995).

The most widely used bactericides are copper-based, and these include copper oxychloride and cuprous hydroxide (Mew & Natural, 1993). The efficacy, relatively low cost and low toxicity to mammals of fixed copper compounds give them an advantage over other chemicals for control of foliar bacterial diseases (Macro & Stall, 1983; Adaskaveg & Hine, 1985).

The mixture of copper with fungicides or antibiotics can result in reduced disease levels due to the evolutionary dilemma presented to the pathogens (Kousik *et al.*, 1996). The combination of copper and mancozeb increases the effectiveness of the copper against both sensitive and resistant strains (Conover & Gerhold, 1982; Kucharek *et al.*, 1986; McCarter, 1992).

CCS is possibly a seedborne disease, and its symptoms are observed in nurseries under warm conditions with high relative humidity. Most of the control measures for this disease therefore rely on treatment of the seed. This can be by physical or by chemical treatment methods.

The aims of this experiment were to determine the effect of different bactericides in controlling CCS, and to assess the phytotoxic effects of the copper-based chemicals.

9.2 Materials and Methods

Preparation of the inoculum

Cultures of the CCS organism (isolate PMK 9608, from Dr Qhobela of the University of Cape Town) were prepared by transferring 48-72 h old cells from nutrient agar into 75 ml nutrient broth in 250 ml Erlenmeyer flasks (refer to 7.2.5). The flasks were incubated at 28°C for 24-36 h on a shaker at 150 rpm. The cultures were then centrifuged in a J2-HS Beckman Centrifuge at a relative centrifugal force of 3000g for 10 mins. The cells were harvested, and suspended in 1/4 strength Ringers Solution. The inoculum concentration was adjusted to 10⁸cfu/ml.

Inoculation

Six to seven week-old cabbage plants (cv. Green Star, lot no. 340301/07030) in size 24 Speedling® trays were inoculated by the pinprick-inoculum saturated cottonwool swab technique in which the leaves were punctured by 8-10 pins, and the inoculum rubbed in to the resultant wounds with a wad of cotton wool soaked with the same inoculum. The same plants were then sprayed evenly with the inoculum by means of an atomizer.

The inoculated plants were placed inside a dew chamber, at a temperature of 28-30°C and RH of 80-100% for 48-72 h. Plants were sprayed again with inoculum 24 h after placement in the dew chamber. After 48-72 h the trays were moved to a greenhouse in which the temperature ranged from 14-34°C and the RH from 50-60%.

Testing the efficacy of bactericides

Seven days after inoculation with the CCS organism, the chemicals shown in Table 9.1 were applied to the seedlings with a knapsack sprayer. The same chemicals were applied to the same plants at weekly intervals, and the disease progress assessed every five days according to a visual rating scale (Appendix 1). The layout of the trial was a randomized block design, and each treatment was replicated four times. The greenhouse temperatures ranged between 14°C and 26°C (winter season) during the period this trial was conducted. Area under the disease progress curve (AUDPC) (refer to 3.2.1) and then ANOVA were run on the disease data.

Table 9.1 Active ingredients and formulations of the different commercial bactericides tested in the seed treatment experiments

Trade Name	Chemical Active Ingredient	Formulation Type	Company	Dosage
Kocide 101	Cupric Hydroxide	Wettable powder	Plaaskem	2 g/l
Kocide 101/ Dithane M45	Cupric hydroxide/ Mancozeb	Wettable powder	Plaaskem/ Zeneca	2 g/l + 2 g/l
Copper Count N	Copper Ammonium Carbonate	Soluble concentrate	Hygrotech Seed	5ml/l
Copper Count N/ Mancozeb	Copper Ammonium Carbonate/Mancozeb	Soluble concentrate/Wettable powder	Hygrotech Seed/ Zeneca	5ml/l + 2 g/l
Shell Copper Hydroxide	Cupric Hydroxide	Soluble concentrate	Shell	2 g/l
Copper Oxychloride	Copper Oxychloride	Wettable powder	Zeneca	4 g/l
Copper Oxychloride/Mancozeb	Experimental chemical	Wettable powder	Zeneca	4 g/l + 2 g/l
Copper Ammonium Carbonate A	Experimental chemical	Soluble concentrate	Ocean Agriculture	5ml/l
Copper Ammonium Carbonate B	Experimental chemical	Soluble concentrate	Ocean Agriculture	5ml/l
Copper Ammonium Carbonate C	Experimental chemical	Soluble concentrate	Ocean Agriculture	5ml/l
Sterisafe 401	Dimethyl Ethyl Benzyl Ammonium Chloride N-Alkyl	Soluble Concentrate	Sterisafe (SA)	1:200
Sporekill	Quaternary ammonium compound	Soluble Concentrate	Hygrotech Seed	10 ppm
Klorman®	Calcium Hypochlorite	Wettable powder	Control Chemicals Deatrick & Associates, Inc.	200 ppm
Lonlife	Experimental chemical	Soluble Concentrate	Kombat	0.3 ml/l

Testing phytotoxicity of the chemicals used for CCS control

Some of the copper bactericides were applied at two and four times the recommended dosages, and their phytotoxic effects evaluated. In these tests, the following copper-containing bactericides were used: Kocide 101, Shell Copper Hydroxide, Copper Count N and Copper Ammonium Carbonate B (CAC B). The greenhouse temperatures ranged between 18°C and 34°C during the time (spring season) this trial was conducted.

9.3 Results

Table 9.2 AUDPC values of crucifer chocolate spot disease (caused by isolate PMK 9608) after treatment with different chemicals expressed as percentage of the control

Treatment	Final disease rating as % leaf area infected	AUDPC	AUDPC as % of control
Control	10.5	187.25 hi	100
Kocide 101 - Mancozeb	7.25	135.6 a	72.4
Kocide 101	7	138.9 ab	74.2
Klorman®	8.75	152.7 bc	81.5
Copper Count N	8	153.4 bc	81.9
Copper Count N - Mancozeb	7.75	153.7 c	82.1
Copper Ammonium Carbonate B	8	155 c	82.8
Shell Cu(OH) ₂	8.75	155.5 cd	83.0
Cu(OCl) ₂ - Mancozeb	8.5	158.15 cde	84.5
Cu(OCl) ₂	8.6	164.75 cdef	88.0
Sterisafe 101	8.75	169.7 defg	90.6
Copper Ammonium Carbonate A (CAC A)	9.9	172 efg	91.8
Copper Ammonium Carbonate C	10	174.8 fgh	93.3
Sporekill	10	183 ghi	97.7
Lonlife	11.5	194.4 i	104.3
F Test	11.2		
LSD(0.05)	14.5		
CV%	1.6		

Treatments with the same letters do not differ significantly at P = 0.05 using Fisher's LSD Test.

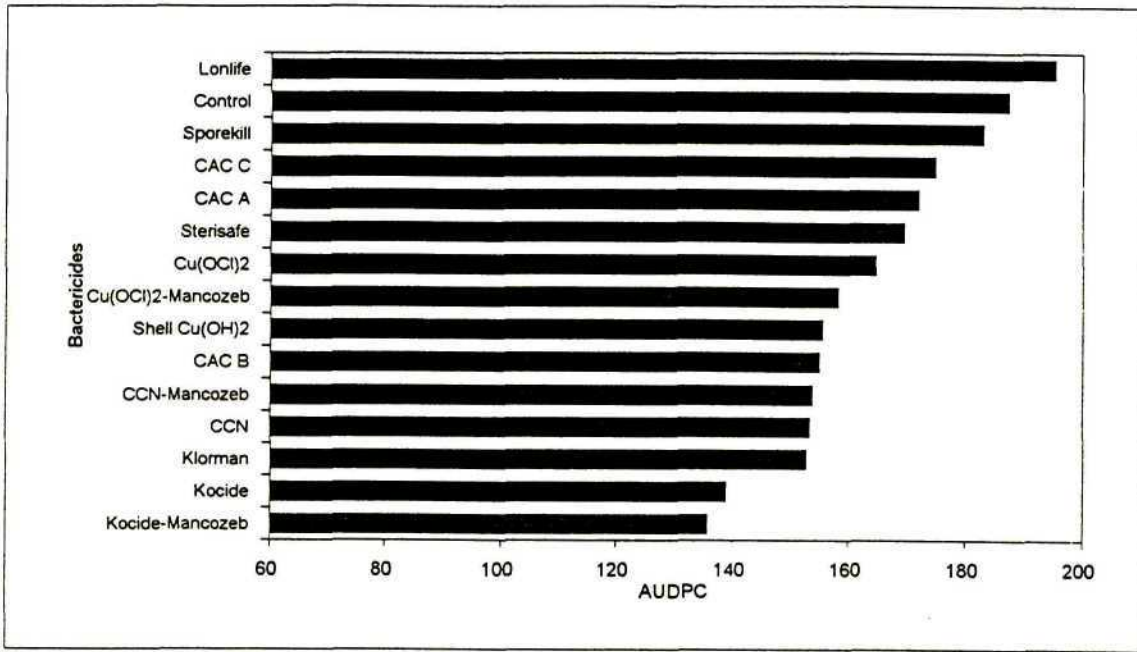


Fig 9.1 The effect of different bactericides on the levels of crucifer chocolate spot disease (caused by isolate PMK 9608)

Table 9.3 Efficacy of different bactericides in the control of crucifer chocolate spot at double the recommended dosage

Treatment	Final disease rating as % leaf area infected	AUDPC	AUDPC as % of control
Control	11	233.7 c	100
Kocide 101	8.4	181.6 a	77.7
Copper Count N	10	204.8 b	85.2
CAC B	10.1	199.1 b	88.5
Shell Cu(OH) ₂	10.3	206.7 b	87.8
F Test	28.3		
LSD (0.05)	10.9		
CV%	2.2		

Treatments with the same letters do not differ significantly at P = 0.05 using Fisher's LSD Test.

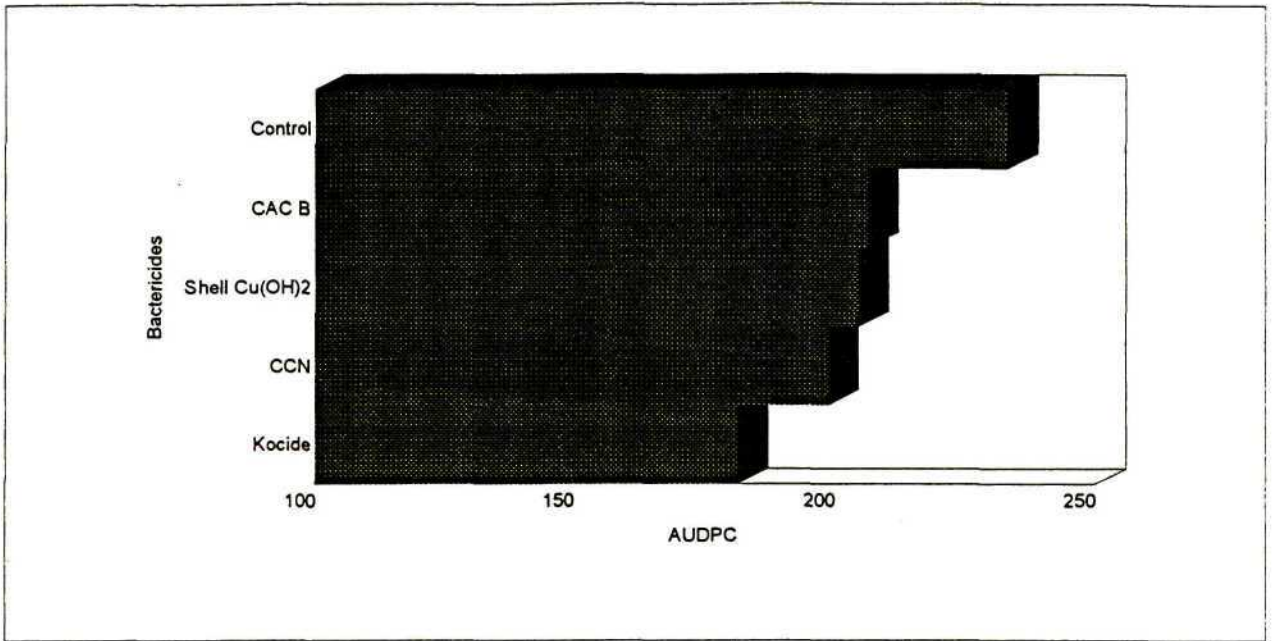


Fig 9.2 The effect of different bactericides applied at double the recommended dosages on crucifer chocolate spot disease (caused by isolate PMK 9608)

Table 9.4 Efficacy of different bactericides in the control of crucifer chocolate spot disease at four times the recommended dosage

Treatment	Final disease rating as % leaf area infected	AUDPC	AUDPC as % of control
Control	10.8	222.2 c	100
Kocide 101	8.5	180.4 a	81.0
Copper Count N	9.25	198.5 bc	92.7
CAC B	10.1	205.9 c	98.2
Shell Cu(OH) ₂	10.5	218.1 ab	89.3
F Test	8.1		
LSD (0.05)	18.1		
CV%	3.7		

Treatments with the same letters do not differ significantly at P = 0.05 using Fisher's LSD Test.

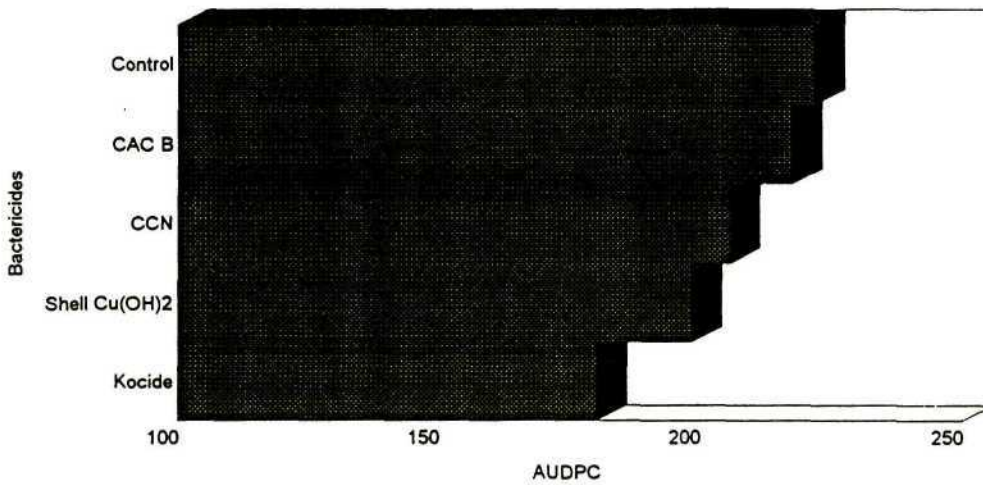


Fig 9.3 The effect of different bactericides applied at four times the recommended dosages on crucifer chocolate spot disease (caused by isolate PMK 9608)

Testing the efficacy of bactericides

The different bactericides tested showed varying levels of CCS disease control (Fig. 9.1). The best control was obtained when a mixture of Kocide 101 and Mancozeb was applied. The quaternary ammonium compounds (Sterisafe 401 and Sporekill) were not as effective as the copper bactericides, and Sterisafe 401 performed better than Sporekill. The least effective of the chemicals was Lonlife. Lonlife is an experimental systemic fungicide/bactericide, and its application resulted in disease levels that exceeded those of the control.

Testing the phytotoxicity of chemicals

For both the 2x and 4x the recommended dosages, the highest level of disease control was obtained when Kocide 101 was applied and the lowest when Copper Ammonium Carbonate B was applied (Figs. 9.2-9.3 and Tables 9.3-9.4). In all the trials, none of the usual phytotoxic effects such as chemical burn, stunting, chlorosis and necrosis were observed. ANOVA showed all the treatments to be significantly different to the control at p=0.05.

9.4 Discussion

Copper bactericides provided better control of CCS disease than did bactericides formulated as quaternary ammonium compounds. Treatments with Kocide 101 resulted in the highest level of disease control for all the trials conducted. Though Kocide 101 and Shell copper hydroxide have a common basic ingredient, $\text{Cu}(\text{OH})_2$, their effects on the control of CCS disease were different (Table 9.2). Similarly, though Copper Count N and CAC A, B, and C have copper ammonium carbonate as the common ingredient, their effects on disease levels were distinctive (Table 9.2). Kocide 101 was supplied as a wettable powder, whereas Shell $\text{Cu}(\text{OH})_2$ was in liquid form. This could have affected the solubility and efficacy of these chemicals. Other factors that could have contributed to the variations in the efficacy of these chemicals include different shelf-life and the presence of different inert compounds.

As all the trials were not synchronized, the influence of temperature, seasonal deviations (winter trials vs spring trials) and inoculation at different physiological stages of plant growth led to varying levels of disease severity developing over the course of the three trials.

The combination of copper based bactericides with mancozeb led to improved performance of the bactericides. This synergistic effect has also been observed with other bacterial spot diseases (Conover & Gerhold, 1982; Adaskaveg & Hine, 1985; Kucharek *et al.*, 1986; Jones *et al.*, 1991; McCarter, 1992). Mancozeb increases the solubility of copper in solution, and the increased solubility improves the efficacy of the copper compounds (Macro & Stall, 1983; Adaskaveg & Hine, 1985). Mancozeb also interferes with the cellular metabolism of pathogens by reacting with the sulfhydryl (SH) groups of amino acids and enzymes thereby affecting the activity of the bacterium (Ware, 1975; Carlile, 1988; Agrios, 1988; Van Emden, 1989).

Bactericides affect disease levels by lowering the apparent infection rate (Vanderplank, 1984). Therefore, effective management of CCS should not rest entirely with the use of foliar chemicals, but, an integrated approach that involves seed treatment, sanitation and resistant cultivars should be utilized.

Increasing the dosage of chemicals up to two or four times the recommended dosage did not lead to phytotoxic effects. However, this is not recommended as it does not increase their efficacy in lowering the severity of CCS, and it is also not economically viable.

Although seasonal variation in the nurseries will influence CCS inoculum levels, application of bactericides was found to be effective in slowing the rate of disease spread.

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CHAPTER 10. SURVEY OF CRUCIFER CHOCOLATE SPOT DISEASE IN THREE PROVINCES OF SOUTH AFRICA

Abstract

A survey of vegetable seedling nurseries in three provinces of South Africa was conducted to determine the prevalence of crucifer chocolate spot. Crucifer chocolate spot was found in 80% of Kwazulu-Natal (KZN) nurseries, but was not detected in either the Mpumalanga or Northern Provinces. Factors that contributed to the high prevalence of crucifer chocolate spot in KZN include the intensity of cabbage seedling production, high relative humidity and warm temperatures. Nutrient stress of seedlings and wounding by pests also contributed to outbreaks of crucifer chocolate spot.

10.1 Introduction

Crucifer chocolate spot (CCS) is caused by a xanthomonad closely related to *Xanthomonas campestris* pv. *campestris* Pammel (Dowson)(Kariem et al., 1995; Laing & Qhobela, 1995; Petersen et al., 1996). It is characterized by dark brown to black circular spots (2-5mm diameter), which may coalesce to form large irregularly shaped lesions. Outbreak patterns of CCS are consistent with hot, humid environmental conditions.

CCS surfaced in several KZN nurseries in the 1990/91 season, resulting in significant financial losses in the production of crucifer seedlings (Laing & Qhobela, 1995). KZN province is situated on the east coast of South Africa, and it faces the Indian Ocean. It has a mean annual precipitation greater than 800mm, and mean annual surface temperatures of 16-22°C (Schulze et al., 1997). Commercial production of crucifer seedlings in KZN is largely through the Speedling® System under shade cloth or plastic covered structures. This results in humid conditions, ideal for the development of CCS.

Control methods that were used by crucifer seedling growers after the initial period of disease outbreak included a variety of chemical and cultural techniques; e.g., use of copper bactericides, hot water treatment of seed and lowering relative humidity (RH) by reducing irrigation frequency. However, these practises did not prevent CCS outbreaks from occurring. Until the end of 1995, there were reports of CCS epidemics occurring in KZN, Western Cape, Northern Province and even in Zambia (Laing⁵, 1996: pers. comm.).

This survey was undertaken to determine the distribution of CCS in some provinces of South Africa, and to identify nursery practises that might promote disease development.

10.2 Research Protocol

Eighteen commercial nurseries and seven small-scale farms were surveyed in the KZN, Mpumalanga and Northern Provinces from December 1995 to December 1996. Seedling growers were asked questions pertaining to the environmental conditions in the vicinity of the nursery, chemicals and fertilizers used in production, source of water and method of irrigation, type of Speedling® Trays and growth media used, and previous incidents of CCS and other diseases (Appendix 2).

Samples from cruciferous plants showing typical symptoms of CCS were collected, and taken back to the laboratory. The causal agent was then isolated according to the technique described by Schaad & Stall (1988). Semi-selective media used were starch-*Xanthomonas* (SX)(Schaad & White, 1974) and nutrient starch cycloheximide antibiotic agar (NSCAA)(Randhawa & Schaad, 1984).

Inoculum was prepared by culturing cells in nutrient broth on a rotary shaker (150 rpm) at 28-30°C. When inoculum density had reached 10^8 cfu/ml, bacterial cells were centrifuged in a Beckman J2-HS centrifuge at 3000g for 10 min, and the resultant pellet resuspended in Ringer's Solution.

⁵Laing, M.D. 1996. Department of Microbiology & Plant Pathology, University of Natal, Pietermaritzburg.

Cabbage (*Brassica oleracea* var. *capitata*) seedlings, cv. Hercules, lot AY010R0 (7-8 weeks old) were then inoculated by pin-pricking and wiping the wounds with a wad of cotton-wool soaked with the inoculum. Following inoculation, seedlings were placed in a dew chamber (28°C, 80-100% RH) for 48 h, and thereafter moved to a greenhouse (18-30°C, 40-60% RH).

10.3 Results

Table 10.1 Areas visited during the CCS disease survey

Areas surveyed	Number of nurseries in the area surveyed	Number of nurseries where CCS disease outbreaks have occurred
<i>Kwazulu-Natal Province</i>		
Camperdown	2	0
Crammond	1	2
Greytown	1	1
Hillcrest	1	1
Ixopo	1	1
Pietermaritzburg	1	1
Richmond	1	0
Tala Valley	2	2
<i>Mpumalanga Province</i>		
Whiteriver	3	0
<i>Northern Province</i>		
Tzaneen/Letsitele/Mooketsi	5	0
<i>Western Cape Province</i>		
George (Laing ⁵ :1997, unpublished)	1	1

⁵Laing, M.D. 1996. Department of Microbiology & Plant Pathology, University of Natal, Pietermaritzburg.

Fig 10.1 (See overleaf)

A map of South Africa with the surveyed areas and areas where CCS outbreaks have been reported

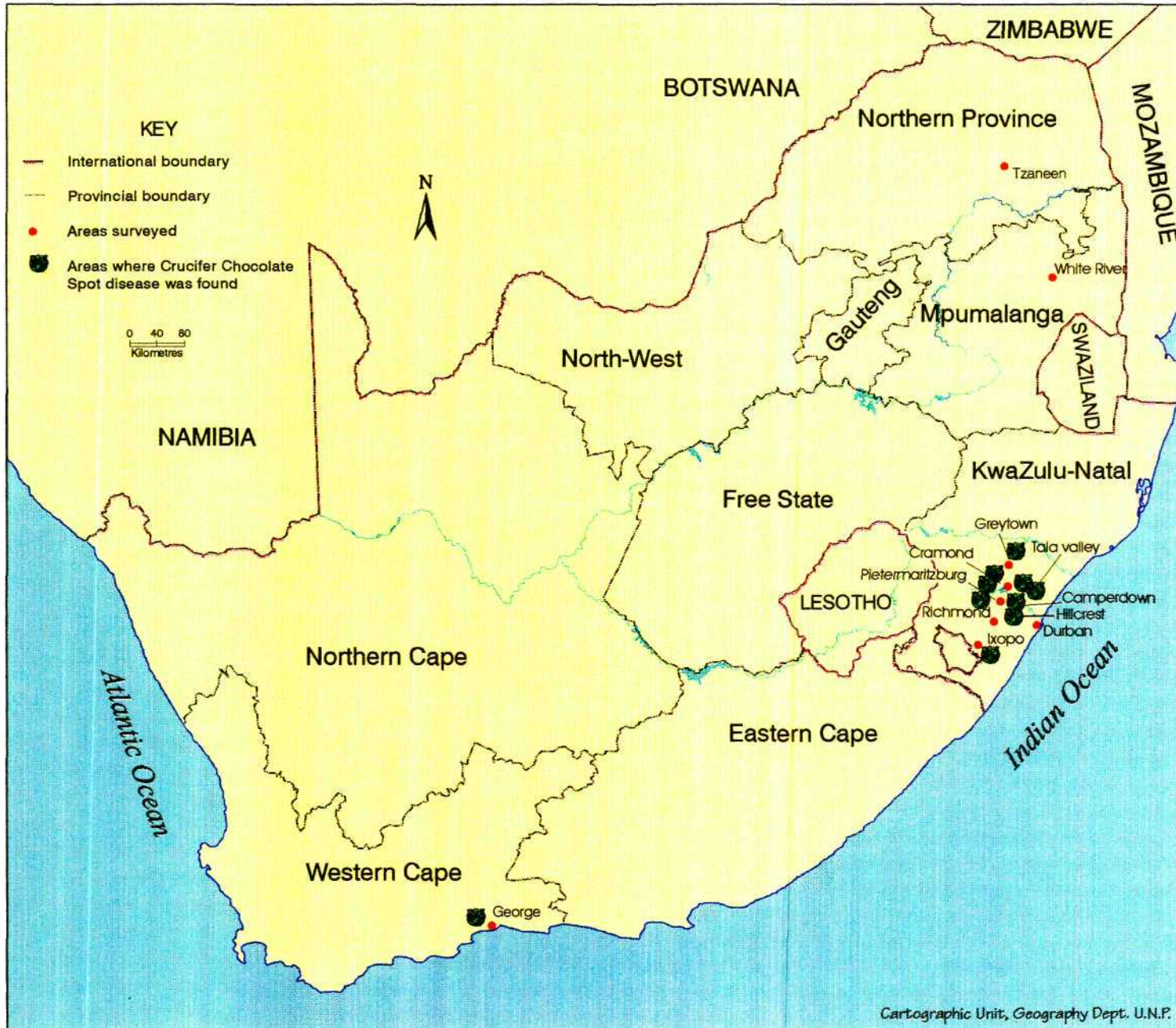
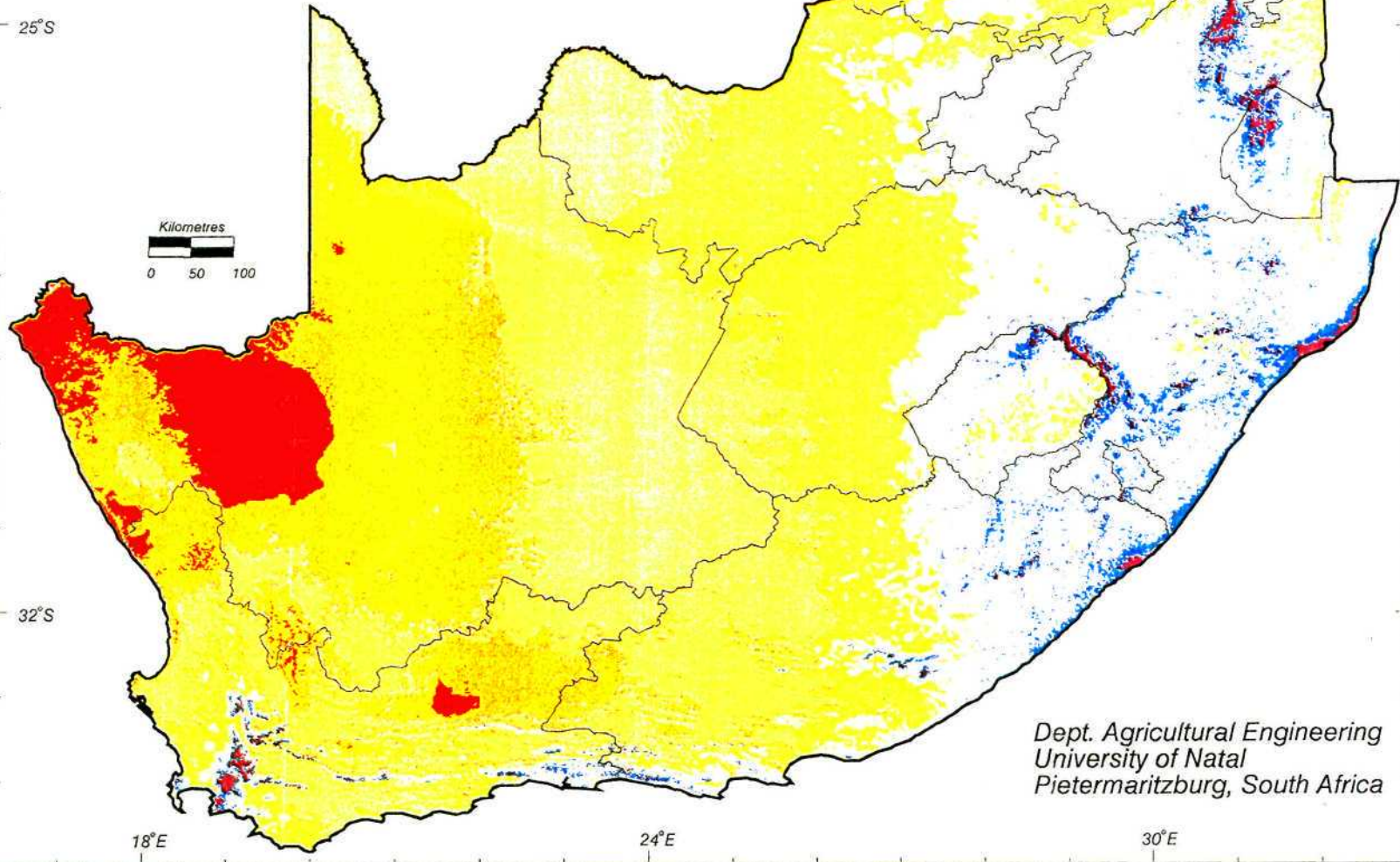
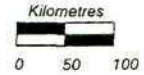


Fig 10.2 (See overleaf)

Mean annual precipitation (mm) in South Africa (Schulze *et al.*, 1997)

**MEAN ANNUAL
PRECIPITATION (mm)**
After Dent, Lynch & Schulze (1989)

25°S



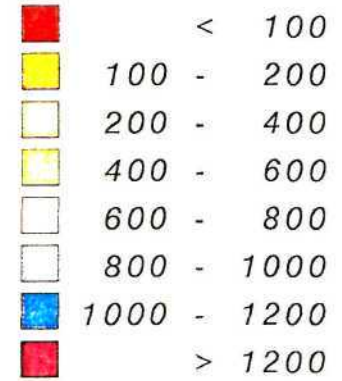
32°S

18°E

24°E

30°E

mm



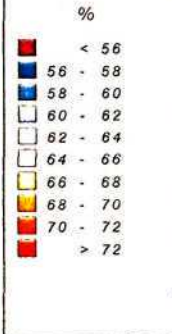
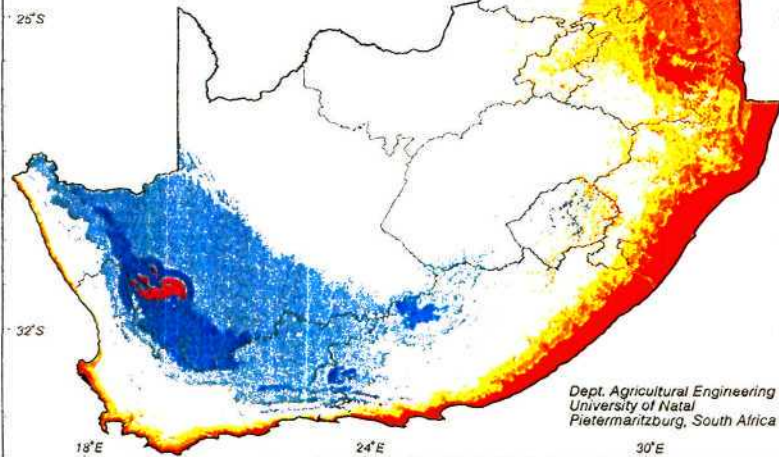
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Pietermaritzburg, South Africa



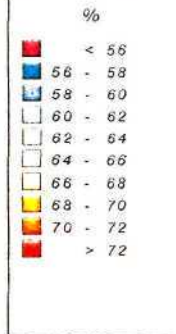
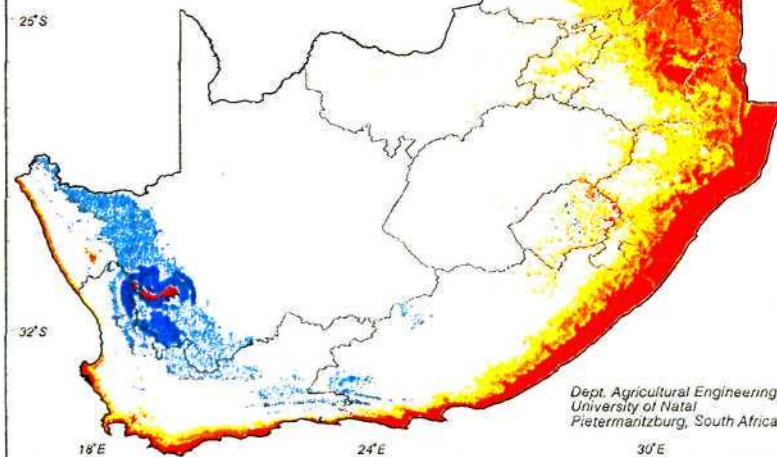
Fig 10.3 (See overleaf)

Daily mean relative humidity (January-April) (Schulze *et al.*, 1997)

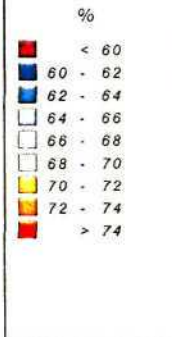
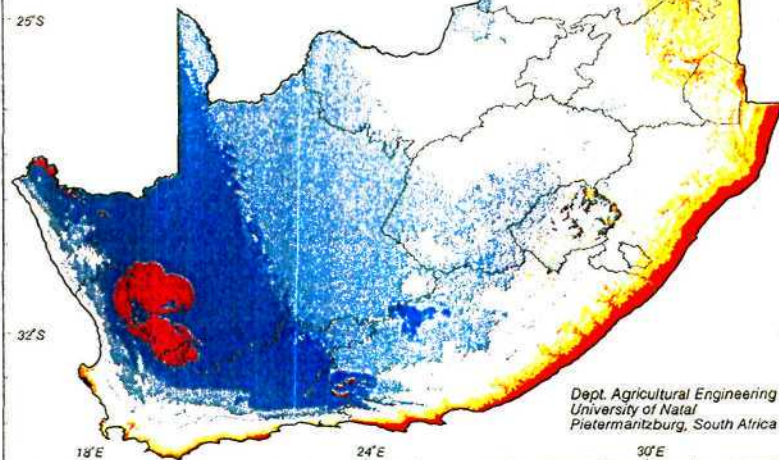
DAILY MEAN RELATIVE HUMIDITY (%)
JANUARY



DAILY MEAN RELATIVE HUMIDITY (%)
FEBRUARY



DAILY MEAN RELATIVE HUMIDITY (%)
MARCH



DAILY MEAN RELATIVE HUMIDITY (%)
APRIL

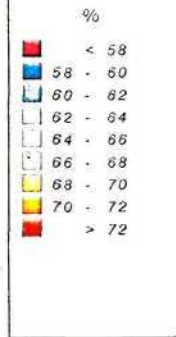
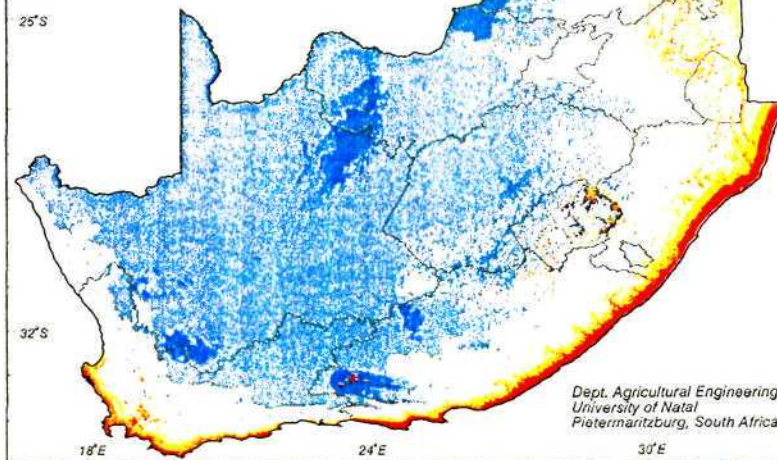
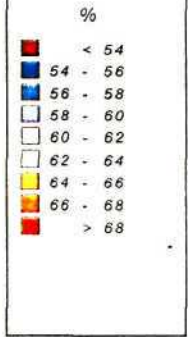
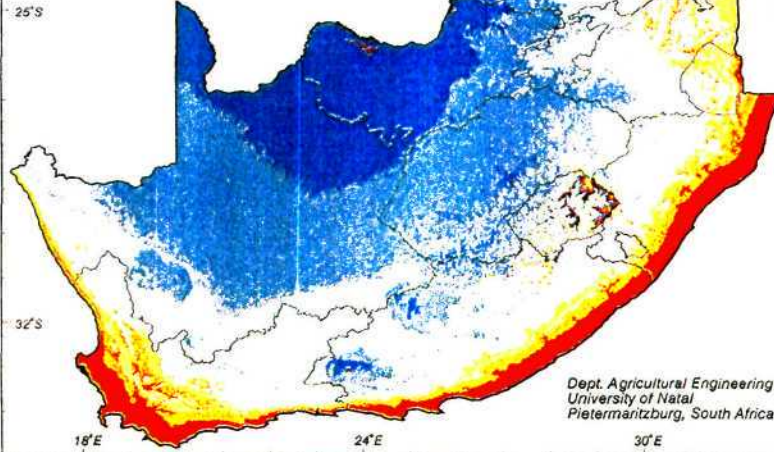


Fig 10.4 (See overleaf)

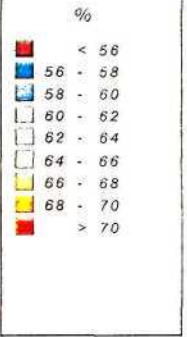
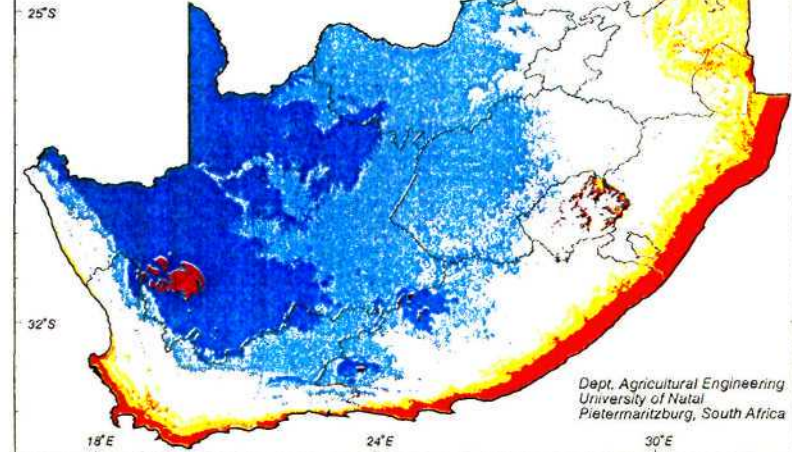
Daily mean relative humidity (September-December) (Schulze *et al.*, 1997)

DAILY MEAN RELATIVE HUMIDITY (%)
SEPTEMBER



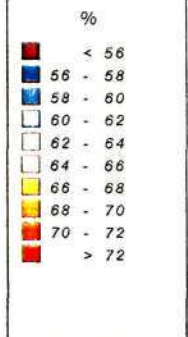
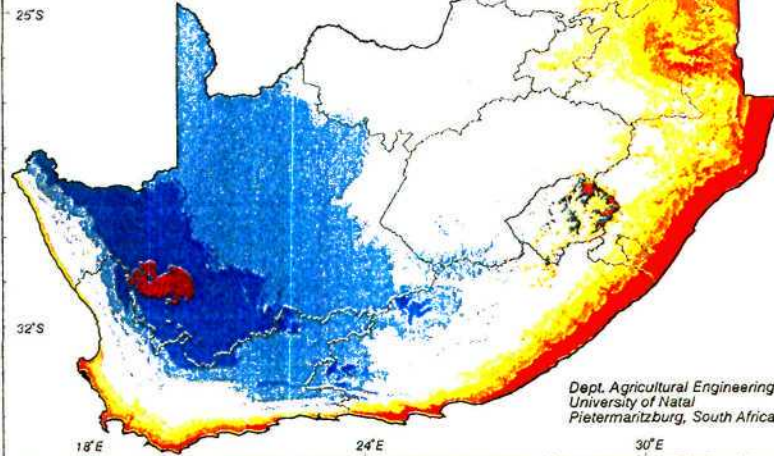
Dept. Agricultural Engineering
University of Natal
Pietermaritzburg, South Africa

DAILY MEAN RELATIVE HUMIDITY (%)
OCTOBER



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University of Natal
Pietermaritzburg, South Africa

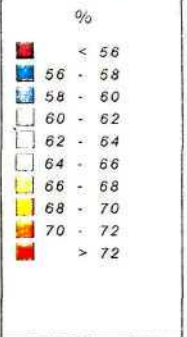
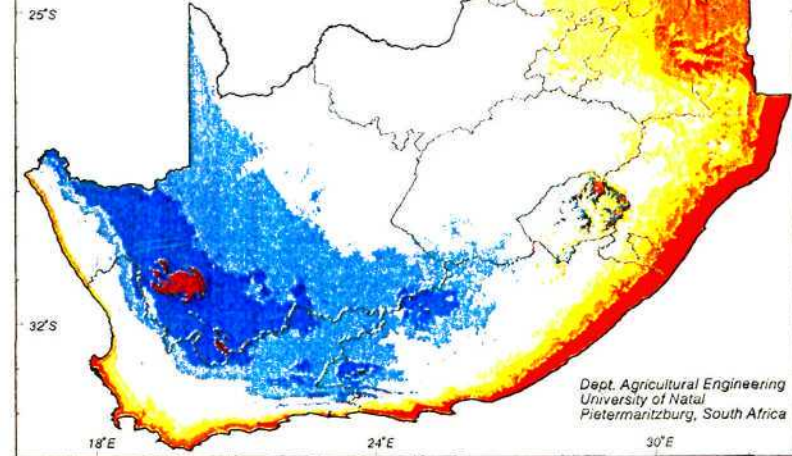
DAILY MEAN RELATIVE HUMIDITY (%)
NOVEMBER



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DAILY MEAN RELATIVE HUMIDITY (%)
DECEMBER



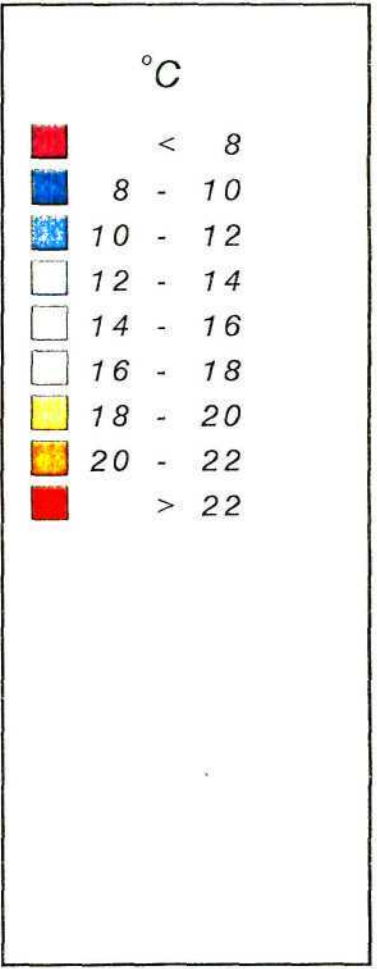
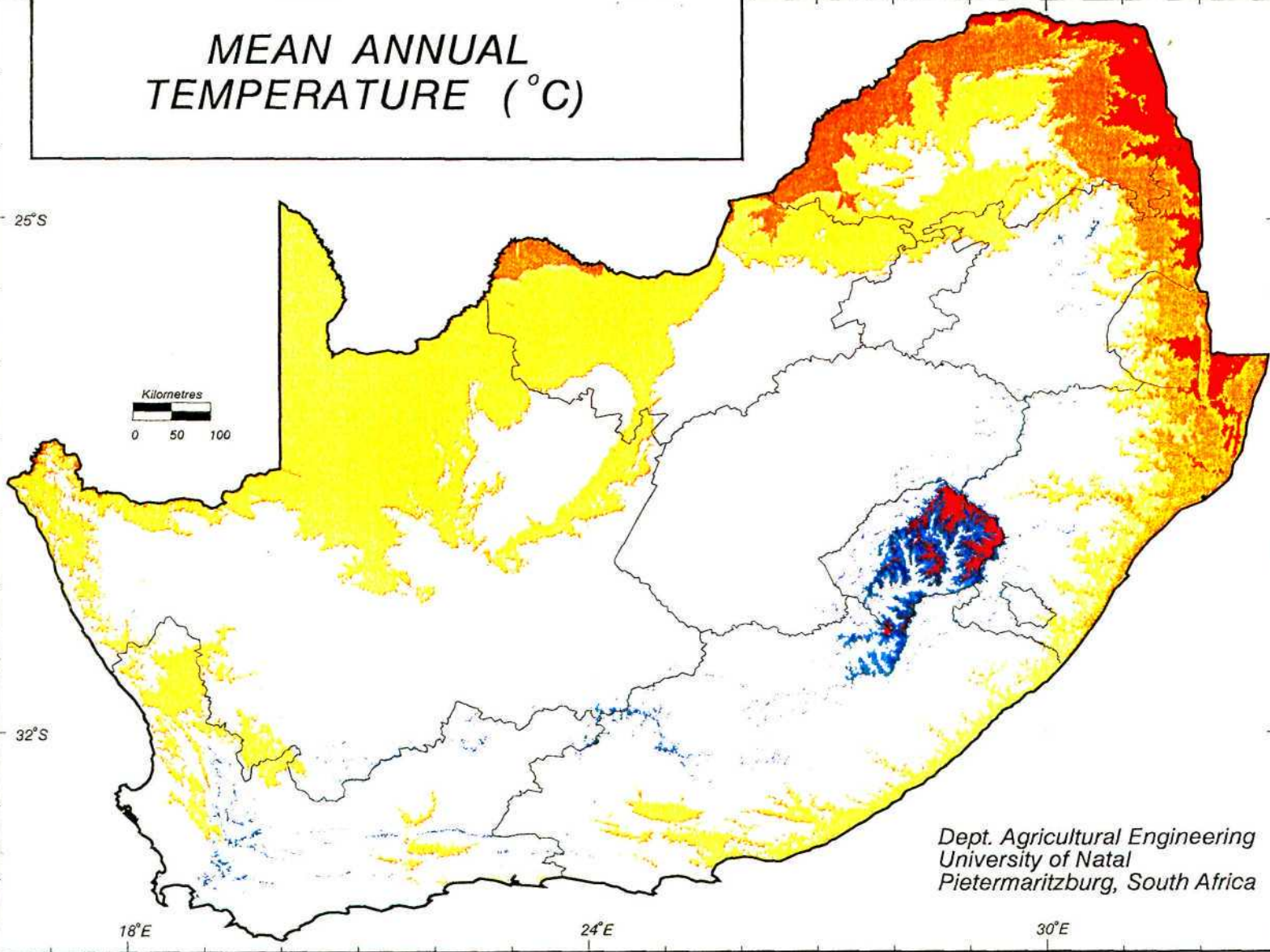
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Fig 10.5 (See overleaf)

Mean annual temperature (°C) (Schulze *et al.*, 1997)

MEAN ANNUAL TEMPERATURE (°C)



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Fig 10.6 Initial symptoms of CCS disease in tunnel-grown cabbage



Fig 10.7 Symptoms of CCS disease in the nursery ten days after the observation of initial symptoms



Fig 10.8 Weeds under tables in one of the commercial nurseries

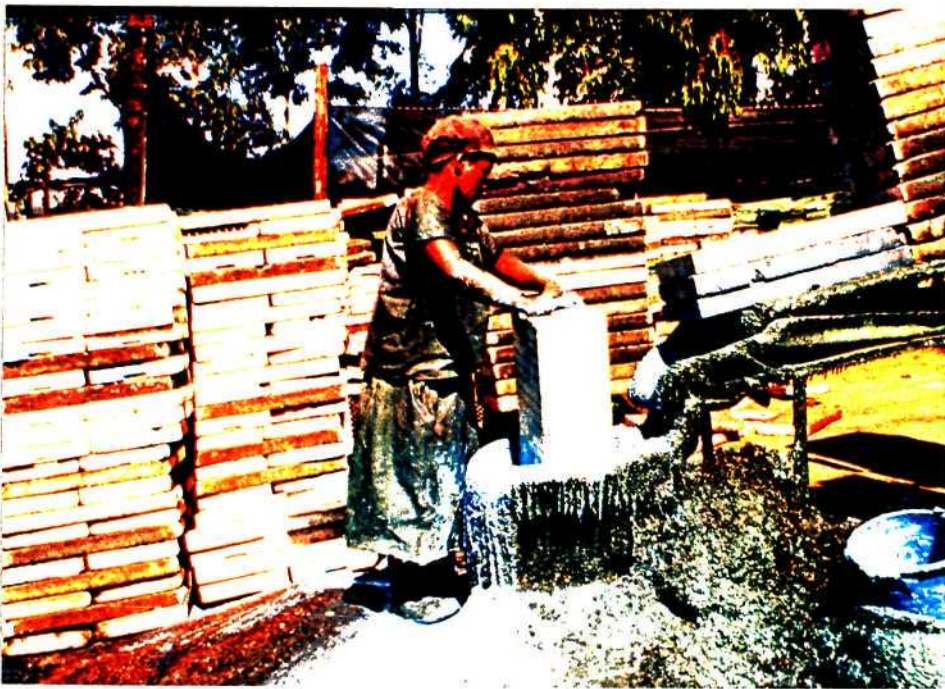


Fig 10.9 Sterilization of trays by Styrodip® at one of the commercial nurseries

No disease was found in the Mpumalanga or Northern Provinces during the survey period 1995-1996 at either nurseries or small scale farms. CCS was found in 80% of the KZN nurseries visited (Table 10.1 and Fig 10.1). Previous reports (before the survey) of CCS outbreaks had been received from nurseries in George (Western Cape), KZN, Northern Province and even in Zambia.

During the survey period Mpumalanga and Northern Provinces accounted for 5-20% of the overall cabbage seedling production in South Africa, whereas 20-90% were produced in KZN Province. Mean annual rainfall, RH, and surface temperatures were also higher in KZN compared to the other provinces (Fig 10.2-10.5).

Surveyed nurseries were circumscribed by different environments including bushveld and thornveld (80%), timber plantations (13%), and sugarcane farms (7%). Covering structures used by seedling producers included shade cloths and plastic shelters while 11% of growers used no covering structures at all.

Thirty-three percent of growers reported using mainly Ocean 3.1.3 (38) fertilizer, whereas other growers used Chemicult, Atlas 315, MAP, Coastal Blend, Spoonspray and own mixtures (of N, P, and K). Fertilizer amounts applied ranged from 100 - 1200 ppm depending on seasonal variations and grower's preferences. However, all producers sometimes kept seedlings at low levels of fertilizer or applied no fertilizer at all during the hardening-off stage. This applied when customers who placed orders were not ready to collect their seedlings, although the seedlings had reached their prime stage.

Growers used various sources of water: springs (7%), rivers (20%), dams (7%) and boreholes (66%). Water quality ranged from clean to very clean. Irrigation methods used included overhead irrigation, boom irrigation, microjets and hand-watering or a combination of these techniques.

Growing media used included Gromed's composted pine bark (40%), Braaks medium (13%), own-mixtures; e.g., peat + vermiculite, composted pine bark + sawdust + clay (47%).

Cabbage diseases and pests of great importance included downy mildew, white rust, damping-off, fungus gnats, caterpillars, thrips and aphids. Diseases and pests commonly associated with CCS outbreaks were downy mildew, fungus gnats, caterpillars, aphids and thrips.

Various types of chemicals were used on cabbage seedlings by different growers, including Dithane®, Copper Count N®, Kocide®, Copper Oxychloride, Bravo®, Previcur®, Ridomil®, Mikal M®, Phosdrin® and Malathion®. Copper sprays were favoured by many growers as a control option for bacterial diseases. No growers were applying any seed treatment.

10.4 Discussion

The occurrence and severity of CCS disease outbreaks were influenced by the geographical location of the nursery. Only nurseries in KZN Province had CCS disease outbreaks during the survey period, whereas CCS disease was not detected from Mpumalanga and Northern Provinces. This was due to the hot, humid (>72% RH) and rainy (>1000 mm per annum) conditions in KZN province (Fig 10.2-10.5, Schulze *et al.*, 1997). Weather conditions (mean RH of 66-70% and annual precipitation of 400-1000 mm) prevailing in the inland provinces, Mpumalanga and Northern Provinces (Fig 10.2-10.5, Schulze *et al.*, 1997), were unfavourable to CCS development. The percentage of cabbage seedling production (20-90% in KZN vs 5-20% in other provinces) accounted for the higher incidence of CCS outbreaks in KZN Province.

Nutrient stress of seedlings exacerbated outbreaks of CCS. This was most evident where hardening-off had occurred, thereby supporting the contention that CCS is a low sugar disease (Chapter 8). Hence the occurrence of CCS can be minimized by not growing seedlings at low fertilizer levels.

Other diseases and pests contributed to weakening the overall defence mechanisms of the seedlings, thereby rendering them more vulnerable to attacks by the CCS pathogen. Invasion of plant tissues by *X. campestris* pv. *campestris* occurs through wounds made by insects, hail or farming equipment (Williams *et al.*, 1972, Williams, 1980, Hunter *et al.*, 1987). Control of pests and protection against unfavourable environmental conditions and diseases should therefore result in reducing the frequency and severity of CCS outbreaks.

Results of the survey suggest that nursery practises; e.g., hardening-off of seedlings, the presence of plant pests and other diseases and abiotic factors contribute to the occurrence and severity of CCS disease. Therefore, regular monitoring of environmental factors (RH, temperature) and general control of plant pathogens can assist in creating conditions unfavourable for the development of CCS.

10.5 References

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CHAPTER 11: GENERAL DISCUSSION AND CONCLUSIONS

Some of the questions that were asked before the commencement of this research were:

- i) Where does crucifer chocolate spot (CCS) disease come from?
- ii) How does the causal agent survive?
- iii) How widespread is the disease?
- iv) Are there crucifer cultivars resistant to CCS?
- v) What precautions could be taken to minimize CCS disease outbreaks?
- vi) What seed treatment methods could be used for seed treatment?
- vii) What bactericides could be applied to minimize losses due to CCS disease?

When CCS outbreaks first occurred in 1991/1992, some growers suspected that the putative pathogen could be seedborne. Seed testing undertaken between 1992-1994 confirmed that some seedlots were infected with a hitherto unidentified pathovar of *Xanthomonas campestris*, which was the causal agent of CCS (Laing⁶ & Qhobela⁷, 1996: pers. comm.). The seed trade then endeavoured to provide CCS free seed and improve the quality of seed sold to growers. This resulted in the decline of CCS disease outbreaks during 1996-1997.

None of the seedlots tested in this project were found to be infected by CCS *X. campestris* following characterization of the putative pathogen by the use of serological techniques, although there was infection by other xanthomonads. Nevertheless, failure to detect CCS *X. campestris* by the techniques used does not mean that the pathogen was not present; it means that it could not be detected by these methods. Thus, the possible role of seedborne inoculum in the epidemiological pathway of CCS cannot be ruled out. Certain seedlots had definitely tested positive for CCS *X. campestris* prior to 1996 (Laing⁶ & Qhobela⁷, 1996: pers. comm.).

⁶Laing, M.D., Department of Microbiology & Plant Pathology, University of Natal, Pietermaritzburg.

⁷Qhobela, M., Department of Microbiology, University of Cape Town, Rondebosch, 7700.

Results of inoculation studies and the disease survey suggested that the pathogen could survive epiphytically and endophytically in living plant material without the manifestation of disease symptoms. However, under environmental conditions favourable for disease development, sudden CCS disease outbreaks were observed. This shows the importance of integrating field monitoring to predict conditions favourable for disease outbreaks.

The survey showed that CCS occurs predominantly in the Kwazulu-Natal (KZN) province, due to the ideal climate and high intensity of cabbage seedling production. However, as reports of CCS disease outbreaks have also been received from other places (Northern Province, Western Province and even Zambia), it can be deduced that the disease could be widespread in South Africa and other parts of the African continent.

The host range of the CCS pathogen was as broad as other xanthomonads, but characterization of the tentative pathogen by the use of biochemical and physiological methods, fatty acid methyl ester analysis and SDS PAGE profiles of cellular proteins (University of Cape Town and Gent University, Belgium) showed differences between the cellular protein profiles of the CCS pathogen and other xanthomonads. The causal agent of CCS was found to be infected by a unique spherical DNA bacteriophage, which never caused lysis of the bacterium (Petersen⁸ & Qhobela⁹, 1995: pers. comm.). Further research needs to be conducted on the role of this virus in the CCS disease. Possibilities that can be explored to explain the taxonomic status of the CCS disease are:

- i) It might be a new pathovar of *X. campestris*
- ii) It might be a new strain of *X.c. pv. campestris*
- iii) It might be that the virus is new, and thereby creating unique disease symptoms.
- iv) It might be a pathotype of *X.c. pv. campestris*, the unique disease symptoms being coded by genes located on the phage genome.

⁸Petersen, Y., Department of Microbiology, University of Cape Town, Rondebosch, 7700.

⁹Qhobela, M., Department of Microbiology, University of Cape Town, Rondebosch, 7700.

Although the focus of this research was on the epidemiology and control of CCS disease, host range studies confirmed the viewpoints of many taxonomists that classification of xanthomonads based on a single phenotypic feature (host-specificity) needs to be re-evaluated.

Artificial inoculation of seedlings with the CCS agent and screening of cultivars for susceptibility was conducted on a large scale using the "pinprick-inoculum swabbing" inoculation technique. Success was dependent on: the age of seedlings at the time of inoculation (6-8 weeks); the nutrient status of the seedlings (should be of low nutrient status); and climatic conditions (28°C, ≥70% RH). However, natural infection in the nursery could occur at RH of ≥40%.

Although a wide range of cultivars could be artificially infected by the causal agent under conditions ideal for CCS, such conditions are not always present in nature. However, these conditions were critical for this study as several attempts to infect the host under less ideal conditions were unsuccessful. Although it was difficult to artificially infect crucifers with inoculum containing less than 10⁷cfu/ml, it is possible that in nature successful infections occur at much lower cell concentrations. There is a general consensus among phyto-bacteriologists that more research needs to be done to understand the differences that occur with regard to the virulence of laboratory cultures and natural isolates.

In order to provide economical and effective solutions to CCS disease problems, interactions between the life cycle of the causal organism (Fig 11.1), the host and the environment must be known. These include knowledge of the origin of the pathogen, mechanisms of dissemination, mode of infection, environmental conditions that favour disease development and overwintering mechanisms. If such information was available control methods could thus be applied at various stages of the disease cycle. Recommendations that can be made in relation to CCS disease management are:

- i) Routine seed testing (by seed companies) for the presence of xanthomonads will help identify contaminated seed lots before they reach the nurseries. Large numbers of seed can be tested economically by soaking the seed in buffer solutions and plating the resultant extracts on semi-selective media.

- ii) Seed treatment will also minimize CCS inoculum levels and hence fewer disease outbreaks will occur. Some treatments that can be used include copper bactericides (at recommended rates), and hot water treatment (50°C for 30 min). The rates that can be used for some copper bactericides are 3 g/l (Kocide 101), 4 g/l (Copper oxychloride) and 5 g/l (Copper ammonium carbonate).
- iii) Control of weeds in the vicinity of nursery tables.
- iv) Sterilization of trays with steam and half-strength Styrodip® will help in minimizing the incidence of CCS.
- v) RH in the greenhouses should be minimized as the CCS pathogen requires high moisture presence for infection to occur. RH can be lowered by not watering before 09h00 and after 15h00 (Laing, 1996: pers. communication). Regular air circulation by the use of fans or situating nurseries at windy sites can also help to keep RH down.
- vi) Increasing the concentration and frequency of fertilizers applied will help to prevent stressing of seedlings and thus minimize the severity of the disease. Significant levels of disease control result from fertilization levels of greater than 400 ppm Ocean 3.1.3 (38) fertilizer.
- vi) Use of copper bactericides; e.g., Kocide 101, also helps in minimizing CCS disease severity (32% disease reduction). Combination of copper bactericides and mancozeb will increase the efficacy of the former.
- vii) Regular monitoring for CCS disease symptoms and seedling stress.

Future research needs to concentrate on the effect of seed treatment on seed of different ages, so as to assess variability in germination percentages of "new" and "old" seed. Research also needs to be conducted on post-treatment of damaged seed, to evaluate if physiological repair mechanisms will result in increased germination and survival.

Other factors for future research on the CCS disease include: identifying the role, if any, of the bacteriophage in the disease; microscopic studies to test the hypothesis that the causal agent of CCS aggregates in the sub-stomatal chambers; breeding of resistant cultivars; assessing the role of weeds as overwintering hosts; monitoring the distribution and severity of CCS in other countries; and, isolation and testing of biocontrol agents to combat CCS.

¹⁰Laing, M.D., Department of Microbiology & Plant Pathology, University of Natal, Pietermaritzburg.

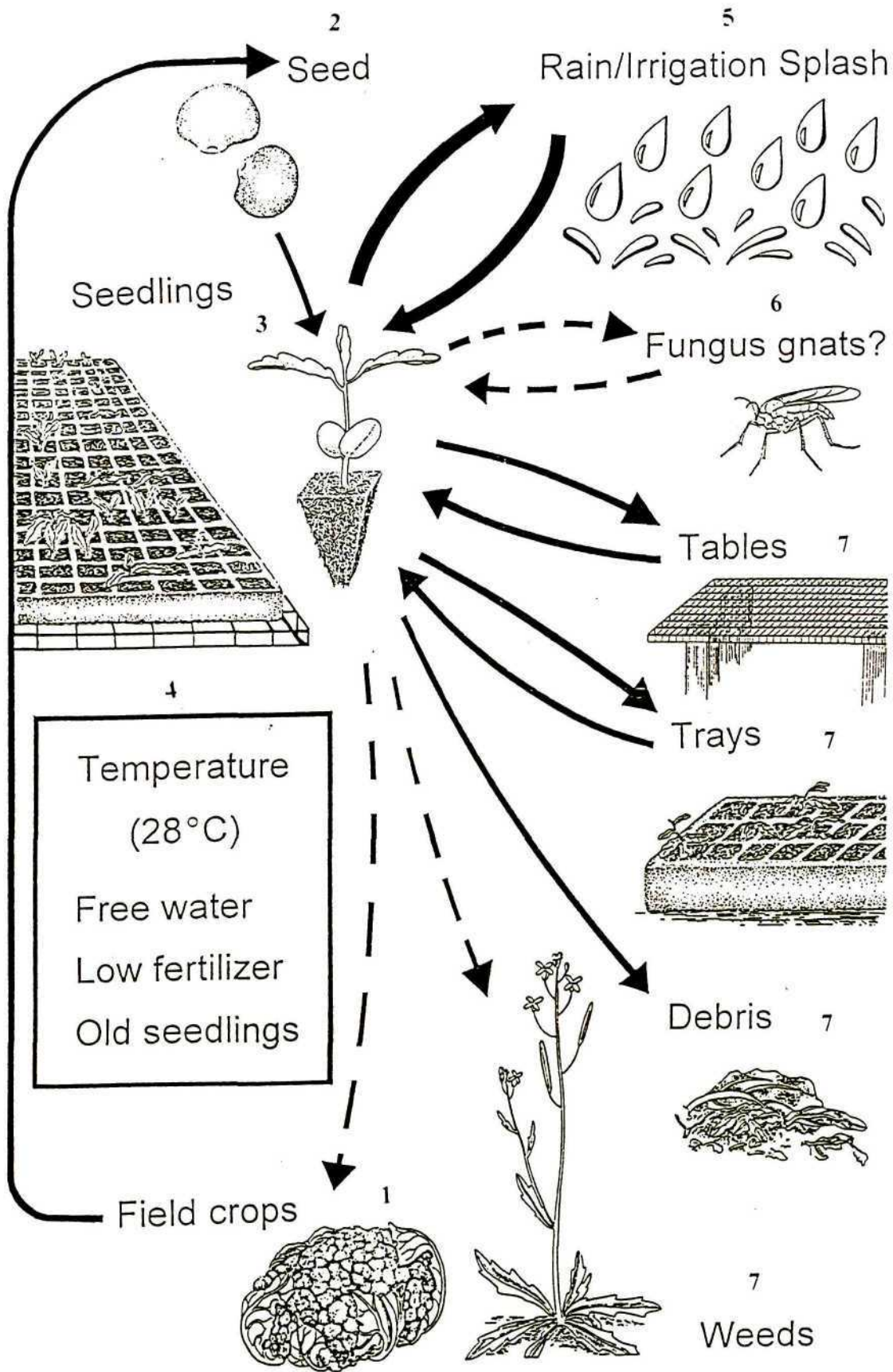
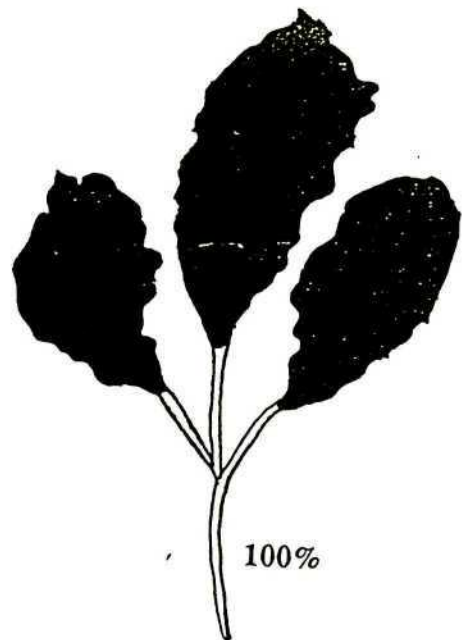
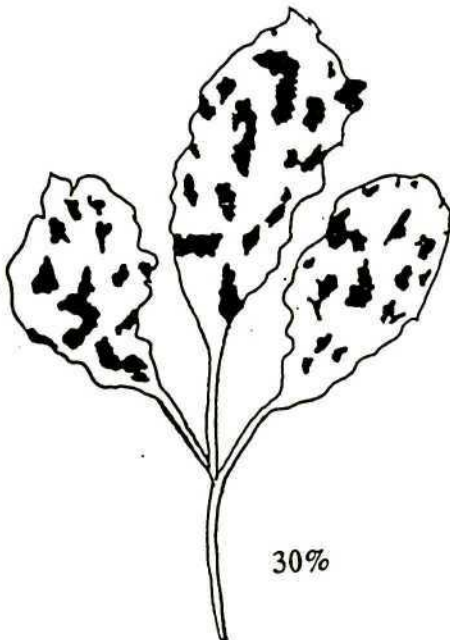
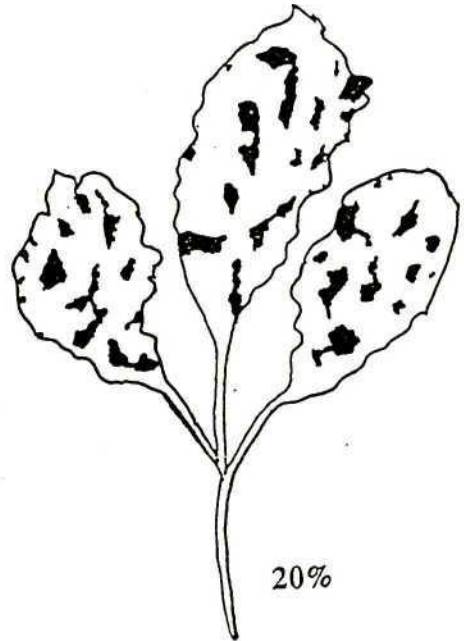
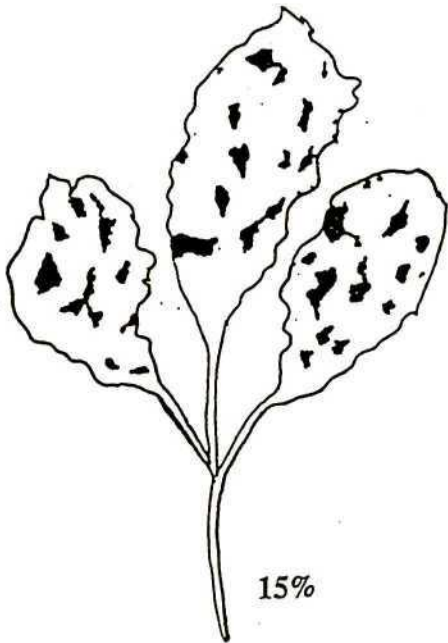
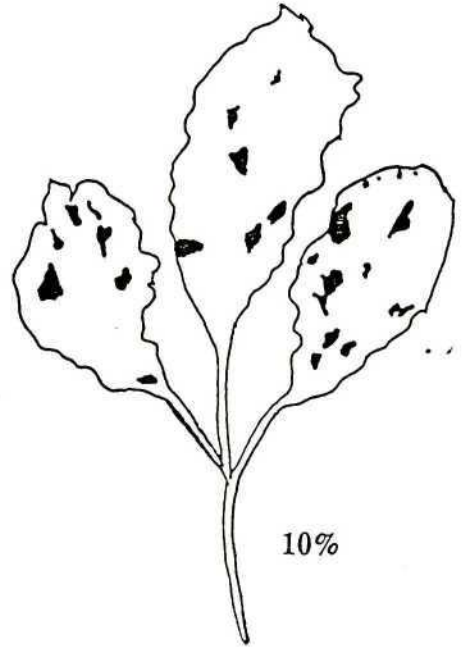
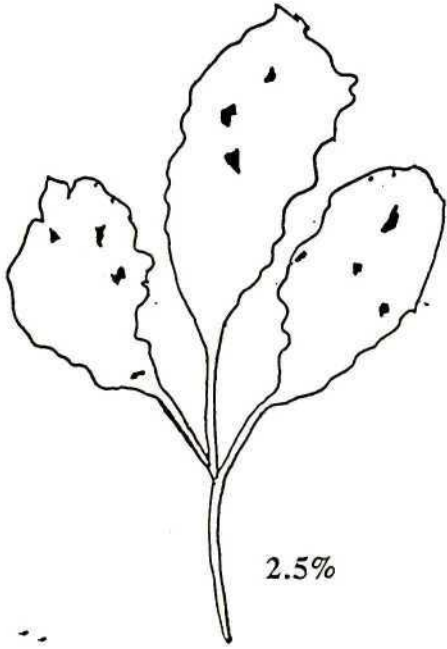


Fig 11.1 An ethograph of CCS disease

Elements of the ethograph:

1. Field crops infected by the CCS pathogen during seed production
2. Dispersal of the CCS pathogen occurs via contaminated seed
3. Seedlings (from infected seed) show typical CCS disease symptoms during favourable conditions
4. Favourable conditions for CCS disease outbreaks include warm temperature (28°C), high RH and nutrient stress
5. Rain/irrigation splash results in disease spread
6. Insects may act as vectors of the CCS pathogen
7. The CCS pathogen overwinters in weeds, debris and speedling® trays

Appendix 1: Visual rating scale for estimating crucifer chocolate spot disease severity
(percentage leaf area infected)



Survey Outline

Nursery's Name

Owner's Name

Owners Wife's name

Manager's Name

Foreman's Name

Address

.

Tel..... Fax

Member of SGASA Yes No

Directions to get there

.

Geog. Location

Altitude

Aspect/Slope: N,S,E,W

Summer Climate: hot/dry hot/wet cool/dry cool/wet

.

Surrounding Environment

Age of Nursery

Structure Used

Table Design wire metal blocks

Average Table Height

Floors earth gravel cinders concrete

Paths earth gravel cinders concrete

Irrigation Type O/head Boom Microjets Hand

Fertilizer used:

Fertilizer application: injector Dosatron Tank-mix Hand .

Fertilizer Level Applied:

Frequency of Application

Water Source river borehole dam

Water Quality v.clean clean dirty v.dirty

Water Treatment none filtered

Primary crops: cabbage %..... Tomatoes %

 gum % pine % Flowers %

.

Trays used for Cabbages: polystyrene plastic
 Cavity Number: 128 200 351
 Tray Sterilization: None Copper-dip steam

Media Used: Braaks SAPPI Hygrotech Gromed coir
 peat own-mix

Cabbage Diseases and pests and importance:

choc spot
 downy mildew
 black rot
 damping off
 pepper spot
 thrips
 aphids
 bagrada bug
 caterpillars
 other pests and diseases

.

Cabbage Sprays & Frequency

Seed Treatment none heat treatment