CHAPTER SIX

SCREENING PLANT EXTRACTS FOR BIOLOGICAL ACTIVITY AGAINST *PENICILLIUM DIGITATUM*, *PENICILLIUM ITALICUM* AND *GUIGNARDIA CITRICARPA*

6.1. Abstract

Garlic (*Allium sativum*) clove and *Coprosma repens* extracts were evaluated for their biological activity against *Penicillium digitatum*, *P. italicum*, and *Guignardia citricarpa* both *in vitro* and on artificially inoculated (in the case of *P. digitatum* and *P. italicum*) and naturally infected (in respect of *G. citricarpa)* Valencia oranges stored at 8±1° C and 90-95% relative humidity (RH) for four weeks. Garlic was evaluated alone or in a mixture with vegetable cooking oil (0.1% v/v). Both garlic and *Coprosma* exhibited varying degrees of antifungal activity against all pathogens, and all concentrations of extracts were significantly effective when compared with the control in checking disease incidence, but were not as effective as the commercial fungicide which gave complete control of both *P. digitatum* and *P. italicum*. Mixing garlic extracts with oil remarkably improved its activity. As a result, the treatment comprising garlic extracts (1 000 ppm) mixed with oil was as effective (100% control) as the fungicide treatment in the control of both *P. digitatum* and *P. italicum*. *Coprosma repens* extract on its own was not as effective and the percentage control achieved varied between 67 and 81%. *In vitro* studies indicate that the mode of action of extracts is inhibition of spore germination and germ-tube development.

6.2. Introduction

Plants contain thousands of constituents and are a valuable source of new and biologically active molecules. Although the potential of these products has long been recognized (Ark and Thompson, 1959), their actual use in this respect is however, still limited. This is particularly true for control of postharvest diseases. Of the several hundred thousand plant species around the globe, only a small proportion has been investigated both phytochemically and pharmacologically (Hostettmann, 1997). Plant extracts are therefore

one of several non-chemical control measures currently being intensively researched for control of plant diseases. Alcohol and water extracts of *Piper betle* L., *Ocimum sanctum* L., and *Citrus limon* (L.) Burm were effective against *Colletotrichum lindemuthianum* (Sacc. & Magnis) in culture, and in checking the incidence and spread of the disease in the field (Amadioha, 1999). Most research with garlic (*Allium sativum* L.) to date has focussed on its medicinal values (Gabe Mirkin, 2001), with few (Pordesimo and Ilag, 1976; Russell and Mussa, 1977; Shashikanth *et al.*, 1981; Garcia and Garcia, 1990; Obagwu *et al.*, 1997; Sinha and Saxena, 1999) reports of its other possible applications such as plant disease control. To date there are no references in the literature on the use of extracts of garlic clove for control of *Penicillium digitatum* Sacc., *P. italicum* Wehmer., and *Guignardia citricarpa* Kiely. Literature abounds on the horticultural values of *Coprosma repens* Hook F. There are however, no references on the use of its extracts in plant disease control.

The aim of this study was therefore to evaluate the efficacy of water and ethanol extracts of garlic clove and *C. repens* for control of *P. digitatum*, *P. italicum*, and *G. citricarpa* and characterize extracts of *C. repens*. The active component of garlic, allicin, is reported to break down easily (Anon., hyp/www/3mistral.co.uk/garlic/allicin). Extracts will therefore be evaluated together with vegetable oil to exploit possible delay of the breakdown and improvement of its biological activity following such a combination. Garlic extracts will be evaluated as a spray application because a dip application might not be economical. Attempts will be made to characterize *C. repens* extracts with a view to identifying the active compound(s).

6.3. Materials and Methods

Twenty-nine plants were screened for their antifungal properties *in vitro* i.e. in microtiter plates (Nunc; AEC-Amersham (Pty) Ltd) at the beginning of this study (unpublished data). Only those that gave promising results in the preliminary studies were evaluated further. In these preliminary studies, garlic and *C. repens* were most promising and were therefore included in further screening.

6.3.1. Fruit

Two orange (*Citrus sinensis* (L.) Osbeck) cultivars, Valencia and Shamouti collected from a commercial orchard at Letaba Estates in the Limpopo Province of South Africa were used in this investigation. No postharvest treatment was applied and fruits were either used immediately after harvest, or stored at 9° C until use (usually no longer than two weeks).

6.3.2. Pathogen

For the source and identity of pathogens used in this study, refer to section 3.3.1 (Chapter Three).

6.3.3. Preparation of Extracts

6.3.3.1. Garlic

Preliminary *in vitro* trials in microtiter plate (result not presented) showed that a higher quantity of powder was required compared to fresh samples to achieve the same level of pathogen inhibition. Only extracts obtained from fresh samples were therefore evaluated further. Similarly, no significant difference in activity was observed between two garlic cultivars (Nootka Rose and Rose du Var). Only Rose du Var was evaluated further.

Preparation of extracts from fresh cloves

Samples were surface disinfested for two minutes in 70% ethanol and washed in three changes of sterile distilled water. One, 3, 5, 7 and 10g sample were separately pulverized in sterile porcelain mortar with a pestle. The resulting pulp was suspended in half the required quantity of solvent (50ml water or 20% ethanol) in 250ml Erlenmeyer flask to form a paste. The paste was agitated for one minute and filtered through sterile cotton wool into a 100ml Erlenmeyer flask and the volume of filtrate made up to 50ml with sterile distilled water or 20% ethanol respectively.

6.3.3.2. *Coprosma repens*

Preparation of extracts from fresh leaves

Leaf samples were washed under tap water for two minutes. Different quantities of samples were separately crushed in solvent (water or 20% ethanol) using a small porcelain mortar and pestle to obtain different concentration of extracts, which was sieved through two layers of cheesecloth to remove uncrushed leaf particles. Samples were sterilized using a 0.22µm pore size syringe driven filter (Millipore). Extracts were either used immediately or kept at 7° C for future use.

Preparation of extracts from dry samples

Leaf samples were washed in tap water for two minutes and blotted dry with tissue paper. Samples were further dried in an oven at 40±1° C for seven days. Dry samples were ground into powder using a small porcelain mortar and pestle. Different quantities (5 000, 10 000 and 20 000 ppm) of powder were suspended in either water or 20% ethanol to obtain different concentrations of extracts, which were sterilized and treated as described in 6.3.3.2.

6.3.4. *In vitro* Screening

6.3.4.1. Characterization of *Coprosma repens* Extracts

The Thin layer chromatography (TLC) and High Performance Liquid Chromatography (HPLC) studies reported below were conducted as preliminary trials to basically identify the family of compounds possibly responsible for the activity of *C. repens* extracts. Detailed analysis to identify the exact compound responsible for extract activity will not be undertaken in this study.

6.3.4.1.1. Chemical analysis of *Coprosma repens* extracts

Determination of total soluble phenolics- (Folin-Ciocalteu's Reaction)

One hundred and seventy microlitres of distilled water was dispensed separately in microtiter plate wells. Next, five microlitre of extract prepared as described in (6.3.4.2) was added to each well, followed by 50 µl of 20% (w/v) sodium carbonate solution. Lastly, 25 µl of Folin and Ciocalteu's Phenol Reagent (Sigma) was added as a colorimetric

indicator to each well. A blank, consisting of identical composition but replacing the sample with water, served as a control. The mixture was properly mixed with a pipette, and the plate incubated at 40° C for 30 minutes. Five wells were used per treatment and the experiment was repeated once. Absorbance was read at 690nm with a Multiskan Ascent V1.24 354-00973 (Version 1.3.1). Data was calculated as equivalent ferulic acid in mg ml^{-1} extract from the standard curve using an equation: $y = 0.9886x + 0.0108$ ($R^2 = 0.997$).

Extraction of free acids

Tetrafluoacetic acid (TFA) 20% was added to extracts prepared as described previously (6.3.4.2) at the rate of 25µl per 1.25 ml extract in an Eppendorf tube in order to obtain a pH of 2.6. An equal volume of diethylether was added and the mixture shaken and allowed to stand briefly to allow separation of fractions, after which the upper phase, was removed with a pipette and placed in a new Eppendorf tube. This procedure was repeated four times. The separated upper phase layers were combined together and diethylether evaporated. One hundred and fifty microlitres of methanol was added and the extracts stored at 5° C until further use.

Extraction of esters

Ammonium sulphate was added to the remaining part of the aqueous phase (6.3.5.1.2) at the rate of 2% w/v (i.e. 0.2 g/1.25 ml extract) and the same volume of ethylacetate was added. The mixture was shaken and the supernatant removed as described above (6.3.5.1.2). The procedure was repeated four times. At the fourth time, 100 µl of methanol was added before the supernant was removed. The supernatants were bulked together and evaporated until dry, after which 150 µl of methanol was added. Extracts were stored at 5° C until further use.

Thin Layer Chromatography

Thin layer chromatography is the simplest and cheapest method of detecting plant constituents. It was therefore used to determine the chemical constituent of *C. repens* in this study. A preliminary trial was conducted to determine the best solvent for use. The solvent systems tested included Hexane/Acetic acid (5:95), Toluene/Acetic acid (4:1), Benzene/Acetic acid/Water (6:7:5) and water alone. Toluene/Acetic acid gave the best separation and was used in subsequent trials. Also both aluminium pre-coated TLC plates

(SIL G-100 UV $_{254}$) Macherey-Nagel and pre-coated glass plates (Silica Gel 60 F-254) Merck were tested to determine which resulted in the best separation. Aluminium was found to be better than glass and was therefore used in subsequent trials. Extracts prepared as described earlier (6.3.3.2) was analyzed on aluminium TLC silica gel plates (10 x10 cm) using the Toluene/ Acetic acid (4:1) solvent system. All assays were run in duplicate. Spots and bands were visualized with a CAMAT 50Hz UV lamp (254 and 366nm). The visibility of compounds on plates was amplified where necessary by flooding developed plates with liquid nitrogen. Three fluorescent bands (1, 2, and 3, Fig. 6.2B) were visible. For identification purpose, band 1 (RF 0.32) was eluted from the TLC plate with methanol (50 µl) for 20 hours in an Eppendorf tube. The mixture was centrifuged for one minute in a microcentrifuge (Helttich Micro Rapid/K) at 7500 x g. The resultant supernant was collected in a new Eppendorf tube and analyzed by HPLC to identify the class of compound present. The second set of TLC plate was used for bioautographic tests.

Bioautography

Chromatogram developed as described above was sprayed with a fungal spore suspension prepared in a nutrient base medium which composed of glucose (30%), (Solution A) with KH_2P0_4 (7g L^{-1}), Na_2HP0^4 (3g L^{-1}), KNO_3 (4g L^{-1}), $MgSO_4.7H_20$ (1g L^{-1}) and NaCl (1g L^{-1}) (Solution B). Solution A and B were mixed in a ratio of 1:6. *Penicillium digitatum*, *P. italicum* and *G. citricarpa* were tested, at a concentration of 10^6 spores ml^{-1}. The TLC plate was incubated for between 48 and 96 hours at 25° C in humidity chambers. Plates were monitored afterwards for germination of pathogen spores. Inhibition zones indicated the presence of active compounds. Where no visible growth was recorded, plates were sprayed with tetrazolium salt (8%). A red colouration indicated respiratory activity taking place indicating that pathogen spores germinated and therefore no active compound was present. An inhibition zone on the other hand, indicated the presence of active compounds.

High Performance Liquid Chromatography (HPLC)

Ten microlitres of *C. repens* extracts prepared as described earlier (6.3.4.2) was injected into the HPLC machine. For qualitative analysis, a gradient elution schedule consisted of an initial one minute run of 10% acetonitrile in ultra distilled water followed by a linear gradient to 50% acetonitrile over 15 minutes at a flow rate of 2 ml per minute. The chromatographic system consisted of Varian 9012 high-pressure pumps (3 phases), a manual injector, an integrated system controller, a MALsil C18 5 micron reverse-phase

analytical column (250 x 4.6 mm, 5µm particle size), and a System Spectra 6000 LP UV diode array detector with an attached analysis computer and data storage system (OS/2 WARP, Thermo Separation Products). Ferrulic and p-coumaric acids were also injected for comparative purposes.

6.3.5. Effect of Extracts on Pathogen Growth *In vitro*

6.3.5.1. Garlic

Two hundred and fifty microlitres of different concentrations (1 000, 3 000, 5 000, 7 000, and 10 000 ppm) of extracts prepared as described earlier (6.3.3.1) was dispensed in microtiter plate wells and inoculated with either *P. digitatum*, *P. italicum*, or *G. citricarpa* spore (1 x 10^6 spore ml^{-1}). The plate was incubated at 25° C for 1, 6 and 12 hours. At each time interval, 50µl extract-spore suspension was pipetted onto 90-mm Petri-plate containing 25ml aliquot of potato dextrose agar (PDA) (Biolab). Inoculated plates were incubated at 25° C for 10 days in the case of *P. digitatum* and *P. italicum*, and 14 days for *G. citricarpa*. Pathogen growth was determined as described earlier (3.3.2.3). Plates inoculated with spores suspended in water served as control. Five plates were used for every concentration tested. The experiment was repeated twice. To determine the effect of treatment on spore development, 100 randomly selected spores were viewed under an inverted microscope (Nikon-TMS, Japan) at 40x magnification and the percentage of spores germinated following 72 hours of incubation at 25° C, recorded.

6.3.5.2. *Coprosma repens*

Extracts prepared as described previously (6.3.4.2) were evaluated for their antifungal activity as described in 6.3.6.1.1. Percentage spore germination was also determined as described in 6.3.6.1.1.

6.3.6. *In vivo* Screening

6.3.6.1. Effect of Extracts on Disease Control

6.3.6.1.1. Garlic

Treatments included; extracts alone, extracts combined with sunflower cooking oil (Black Cat), extracts combined with fruit wax (Polyorange at 0.1% vol/vol), oil alone and wax alone. The choice of "Black Cat" was based on results of a preliminary *in vitro* trial (data

not presented) to test the effects of different sources of vegetable oil on germination of *P. digitatum* spores. Although all oils were effective in inhibiting spore germination, "Black Cat" was cheaper and more readily available. *Guignardia citricarpa* was not included as a treatment because of logistic problems. *Penicillium digitatum*, and *P. italicum* inoculated fruits were sprayed about six hours after inoculation with one of five treatments prepared as described earlier using a spraying bottle (Efekto). The control consisted of fruits sprayed with sterile distilled water or commercial fungicides (see section 4.3.6). Treated fruits were stored in cardboard boxes at 9 °C, and 90 to 95% RH for four weeks, and assessed for decay symptoms as described previously (4.3.6).

Effect of treatment on pathogen development on fruit

To determine the effect of treatments on the development of *Penicillium,* peel pieces were taken from the inoculation point at 36-48 hours after inoculation with a no.1 cork borer. Six peel pieces; namely two per fruit were taken and bulked. The peel macerate prepared in 10 ml sterile distilled water was sieved through two layers of sterile cheesecloth. One hundred spores were observed under the inverted microscope (Nikon TMS) for germination

6.3.6.1.2. *Coprosma repens*

Unlike garlic, *C. repens* was evaluated as a dip treatment. This is because the plant is readily available and large volumes of extracts could easily be prepared. Fruits artificially inoculated with *Penicillium* as described in 4.3.6 were immersed for one to two minutes in different concentrations of extracts prepared as described previously (6.3.4.2). Treated fruits were dried and then waxed. Storage and disease assessment was done as described in 6.3.7.1.1 and 4.3.6 respectively. In the case of *G. citricarpa*, old lesions on naturally infected fruits were marked as described earlier (4.3.9) and fruits sprayed as described above. Efficacy of treatment was based on the number of new black spot lesions appearing following treatment. Thirty fruits were used per replicate and each treatment was replicated three times. Treated fruits were stored at 9 °C for three weeks, and 23±1° C for one week. The experiment was repeated twice.

Effect of storage duration and storage temperature on efficacy of *Coprosma repens* extracts

Extracts prepared as described in 6.3.4.2 were stored at 23±1° C and 9 °C for 30, 60, and 90 days. Extracts were evaluated at each time interval (i.e. 30, 60 and 90 days) as described earlier (6.3.7.1.2) for its activity against *P. digitatum*, *P, italicum*, and *G. citricarpa*.

6.3.7. Statistical analysis

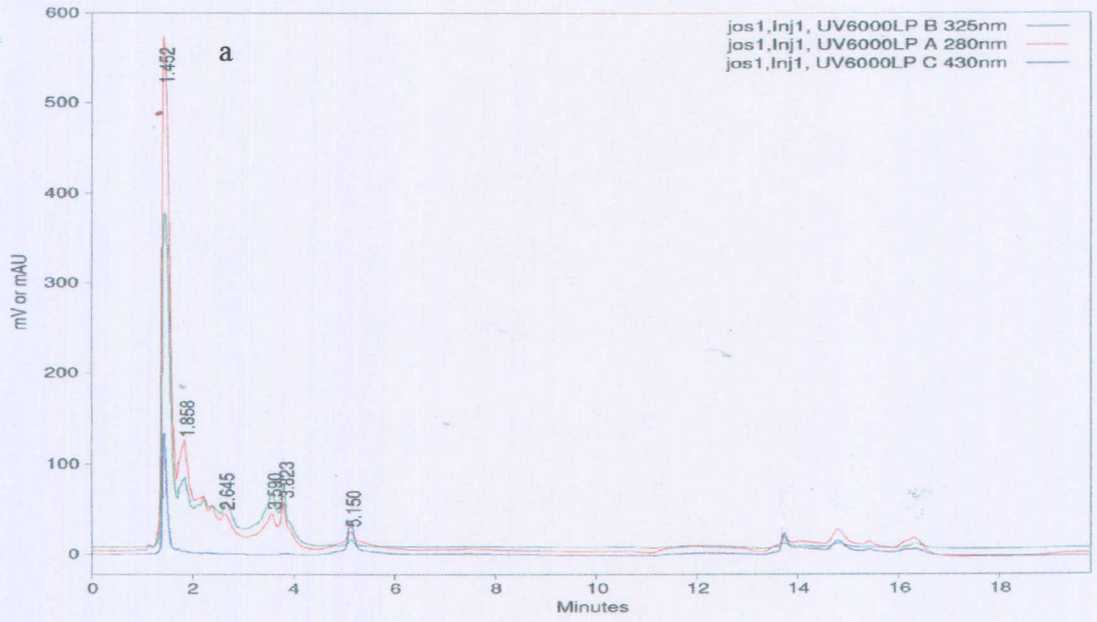
Data was statistically analysed using the GenStat statistical program. Two-way analysis of variance (ANOVA) was used to test for differences in average means between treatments. Treatment means were separated using Fishers' protected t-test at a 5% level of significance.

6.4. Results

6.4.1. *In vitro* Screening

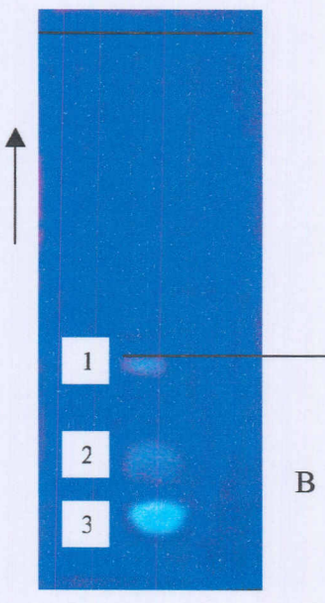
6.4.1.1. Characterization of *Coprosma repens* Extracts

Results of the chemical analysis of extract constituents and the subsequent anti-microbial activity are summarized in Figs. 6.1-6.3. The HPLC run of crude extract indicated one major peak (a0 and five minor peaks in the chromatogram (Fig.6.1A). The extract chromatograph on silica gel using Toluene/ Acetic acid (4:1) as solvent, showed two prominent blue fluorescent spots at 350 nm wavelength (spot 1 and 2, Fig. 6.1B), with Rf values of 0.32 and 0.11 respectively. This solvent only allows the migration of the free acids. The third spot indicated as 3 in Fig 6.1B represents the loading spot of the crude extract and showed a milk-coloured fluorescent. When spot 1 (Rf 0.32) was extracted in methanol and further characterized by HPLC, results obtained (Fig 6.1C) indicated one peak in the chromatogram which corresponded to the peak "a" in Fig. 6.1A. The spectrum (Fig.6.2) and the fluorescence and Rf value of the band 1 and therefore peak "a" are similar to those obtained for the hydroxycinamic acids and so confirm the allocation of the compound (peak A) to this family. Results obtained from the bioautographic test indicated that free acid extracts were effective in inhibiting spore germination at all concentration tested (Fig.6.3). Spot 2 also has a similar Rf to those recorded for p-coumaric acid and is

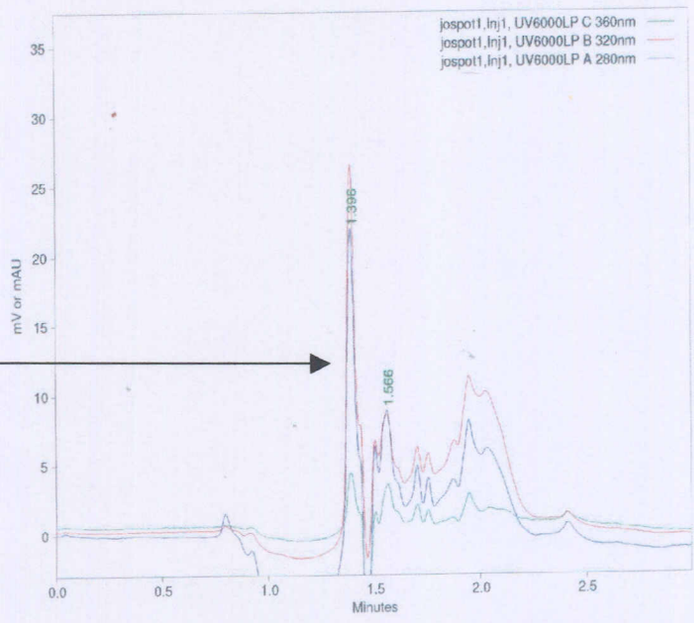


A

Toluene/AA



B



C

(A) HPLC chromatographic run of crude extract (B) TLC chromatographic run of crude extract (C) HPLC chromatographic run of fraction 1 ran at 320nm (see arrow). 1 and 2 represent different fractions (bands), from extract, fraction 3 represent injection point of crude extract.

Fig. 6.1 Chemical screening of *Coprosma repens* leaf extracts using High Performance Liquid Chromatography and Thin Layer Chromatography

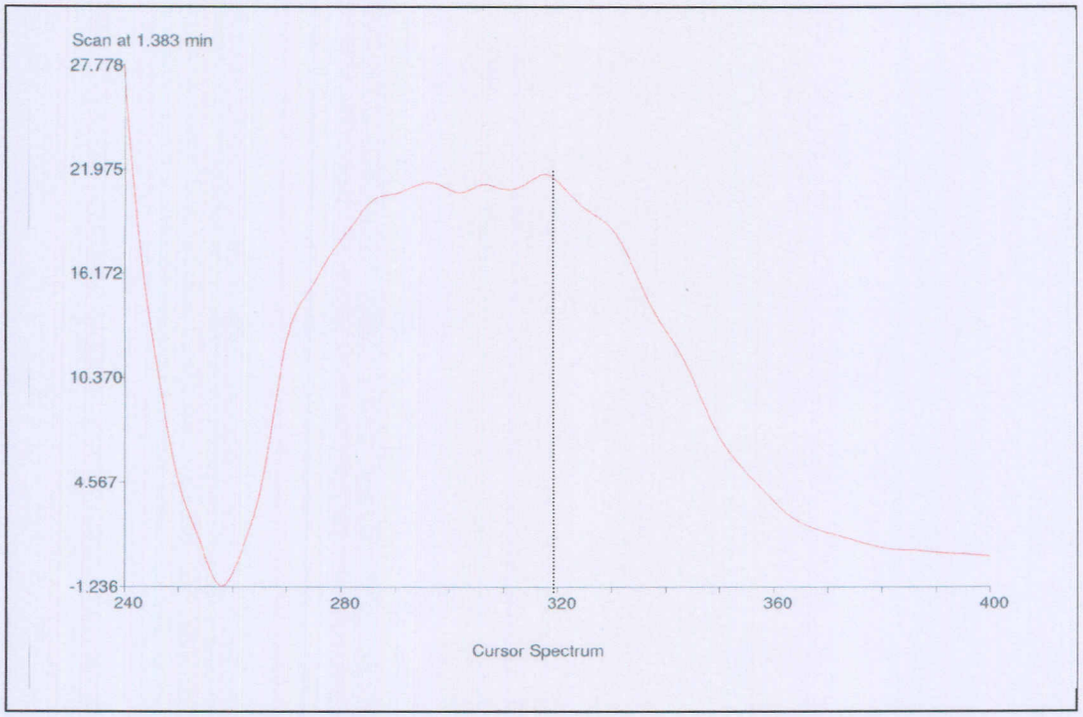
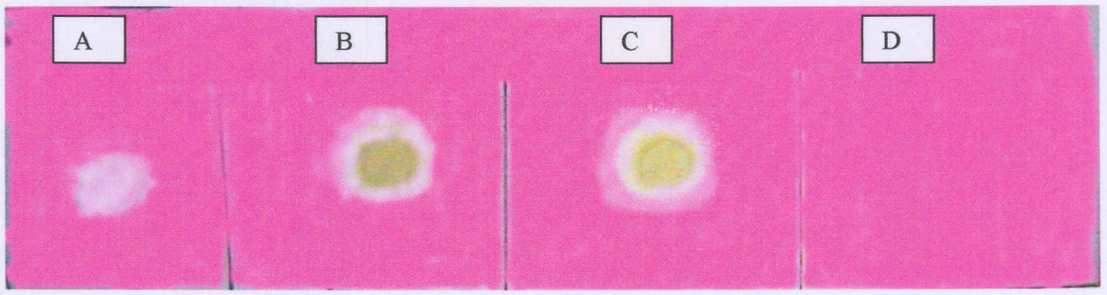


Fig. 6.2 Chromatographic spectrum of "band 1" corresponding to compound "A" (see Fig. 6.2A & B).



A, B, C, and D represent 25, 50, 100 and 0% (control) concentration of free acids White zones indicate inhibitory activity of compound.

Fig. 6.3 TLC bioautographic assay showing effects of free acids extracted from *Coprosma repens* leaf on germination of *Penicillium digitatum* spores after 72 hours of incubation at 25° C.

possibly a compound from the hydroxycinamics. The pool of soluble esters was not effective and results are therefore not presented.

6.4.1.2. Effect of Extracts on Pathogen Growth *In vitro*

6.4.1.2.1. Garlic

Garlic clove extracts had a significant effect on the growth of all three pathogens screened. The concentration of extracts had a significant effect on its efficacy, and all concentrations were effective when compared with the check in the control of pathogen growth. Results presented in Table 6.1 shows a proportional decline in fungal colony diameter with increasing extract concentration and duration of spore suspension. Extracts were more effective in the control of *G. citricarpa* than *P. digitatum* and *P. italicum*. As a result, all concentrations above 1, 000 ppm completely inhibited the growth of the pathogen *in vitro*. Extracts gave better control of *P. italicum* compared to *P. digitatum*. When spores were inoculated on PDA within one hour of suspension in extracts, none of the concentrations completely inhibited the growth of both *P. digitatum* and *P. italicum*. After three hours of suspension, however, complete inhibition of growth was observed for both pathogens at 7 000 and 10 000 ppm respectively.

Extracts had a negative effect on spore germination. Results presented in Table 6.2 show a progressive decline in percentage spores germinated with increasing concentration of extracts. No visible growth of both *P. digitatum* and *P. italicum* was observed in treatments comprising 7 000 ppm and above, and in all concentrations in the case of *G. citricarpa*. Results presented in Table 6.2 however, indicated that some spores did germinate at these concentrations.

6.4.1.2.2. *Coprosma repens*

Data presented in Table 6.3 shows that the *C. repens* extract contains a valuable amount of phenolic compounds. It was observed that over 80% of phenolic compounds were extracted when the suspension was allowed to stand for six hours or more.

Table 6.1 Effects of aqueous garlic extracts on the growth of *Penicillium digitatum, Penicillium italicum*, and *Guignardia citricarpa* on potato dextrose agar incubated for eight days at 25±1° C.

Concentration of garlic extracts ('000 ppm)	Time of spores suspension in extract (h)/Fungal colony diameter (mm) *					
	1 h			3 h		
	Pd [x]	*Pi* [y]	Gc [z]	*Pd* [x]	*Pi* [y]	Gc [z]
0	80.1 a	69.5 a	15. 0 a	77.2 a	51.1 a	16.5 a
1	61.1 b	52.2 b	0.0 b	42.2 b	31.3 b	0.0 b
3	53.0 c	49.4 c	0.0 b	30.1 c	22.1 c	0.0 b
5	35.3 d	34.7 d	0.0 b	10.0 d	5.2 d	0.0 b
7	30.9 d	30.1 d	0.0 b	0.0 e	0.0 e	0.0 b
10	24.4 e	16.5 e	0.0 b	0.0 e	0.0 e	0.0 b

[x] represent *P. digitatum*; [y] represent *P. italicum*; [z] represent *G. citricarpa*

* Mean of five replicates. Means having the same letter in the same column are not significantly different according to Fishers' protected t-test (P = 0.05).

Table 6.2 Effects of aqueous extracts of garlic and *Coprosma repens* on germination of *Penicillium digitatum, Penicillium italicum*, and *Guignardia citricarpa* spores.

Treatment	Pathogen/Percentage spore germination [a]		
	Pd[x]	Pi[y]	*Gc*[z]
1 000 ppm garlic	43	38	6
3 000 ppm garlic	20	30	0
5 000 ppm garlic	12	30	0
7 000 ppm garlic	10	10	0
10 000 ppm garlic	0	10	0
2 5 00 ppm *C. repens*	30	21	8
5 000 ppm *C. repens*	5	20	0
Water control	68	48	25

[x] represent. *P. digitatum*; [y] represent *P. italicum*; [z] represent *G. citricarpa*

[a] Mean of five replicates

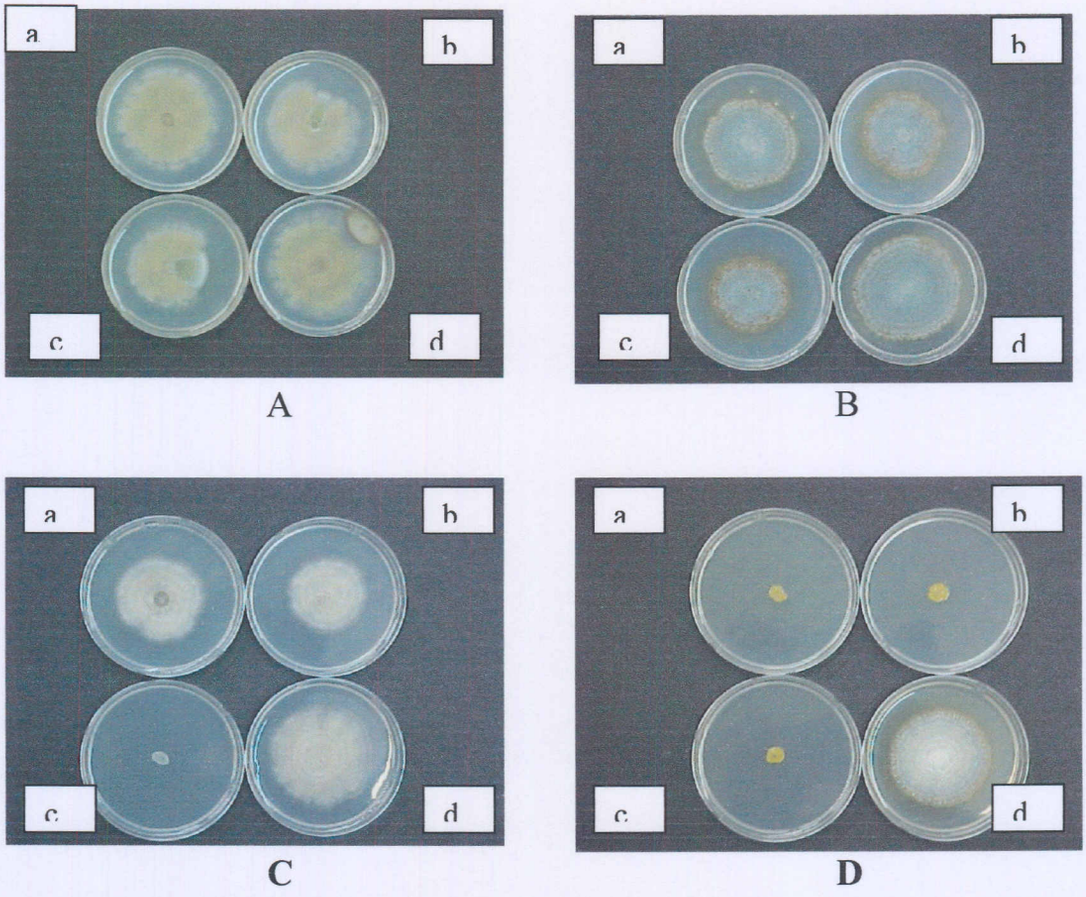


Fig. 6.2 Growth of *Penicillium digitatum* (A and C) and *Penicillium italicum* (B and D) on potato dextrose agar after spore suspension in 3 000 (a), 5 000 (b), 7 000 (c), and 10 000 ppm (d) aqueous garlic extracts for one hour (A and B), and three hours (C and D), and 10 days of incubation at 25° C.

Table 6.3 Quantity of soluble phenolics extracted from *Coprosma repens* leaf powder at each extraction using 20% ethanol as solvent.

Number of extractions	Total soluble phenolics (equivalent ferulic acid mg ml^{-1})	Standard error
1	2.855	0.384
2	2.115	0.304
3	0.775	0.244
4	0.446	0.076

Coprosma repens extracts have great potential for control of all three pathogens screened in the present study. From the results presented on Table 6.4 it is clear that extracts were effective when compared with the water check in inhibiting pathogen growth. A progressive decline in fungal colony diameter was recorded with increasing extract concentration. Extracts were more effective in the control of *G. citricarpa* compared to *P. digitatum* and *P. italicum*. When assayed against G. *citricarpa*, complete inhibition of growth was observed at concentrations above 5 000 ppm, unlike *P. digitatum* and *P. italicum* where none of the concentration tested completely inhibited pathogen growth. The growth of both pathogens at 20 000 and 10 000 ppm did not differ significantly.

6.4.2. *In* vivo Screening

6.4.2.1. Effects of Extracts on Disease Control

6.4.2.1.1. Garlic

All concentrations of extracts were effective compared with the water check in controlling the incidence of both green- and blue mold. They were however, less effective than the fungicide treatment, which gave complete control of both diseases (Table 6.5). The results also show that extracts were more effective on Valencia than Shamouti. A significant increase in the biological activity of extracts was observed when treatments were combined with oil. Consequently, the treatments comprising 1 000, 3 000, and 5 000 ppm combined

Table 6.4 Effect of ethanol extracts of *Coprosma repens* on the growth of *Penicillium digitatum* and *Penicillium italicum on* potato dextrose agar after eight days of incubation at 25±1° C or 14 days in the case of *Guignardia citricarpa*.

Treatment	Fungal colony diameter (mm) *		
	Pd [x]	Pi [y]	Gc [z]
CP 20 000 ppm	5 a	10 a	0 a
CP 10 000 ppm	6 a	10 a	0 a
CP 5 000 ppm	10 b	20 b	8 b
20% Ethanol	21 c	28 c	21 c
Water control	57 d	69 d	30 d

[x] represent *P. digitatum*'; [y] represent *P. italicum*; [z] represent *G. citricarpa*.

* Mean of five replicates. Means having the same letters in the same column are not significantly different according to Fishers' protected t-test (P = 0.05).

with oil (0.1%), for example were as effective (100% control), as fungicide in the control of green mold. The increased activity was more obvious on Valencia and the same level of control was not achieved when extracts were combined with wax. A comparatively higher level of disease incidence was recorded on treatments involving ethanol extracts relative to water extracts (Table 6.5).

Results presented in Table 6.6 shows that treatments were more effective in controlling blue than green mold. As a result, the treatments comprising 3 000 and 5 000 ppm plus oil were as effective as the fungicide treatment which gave complete control of blue mold on both Valencia and Shamouti. This was in contrast to green mold where complete control with the above treatments was achieved only on Valencia.

Table 6.5 Effects of garlic extracts on their own, or in combination with vegetable oil, or fruit wax, on control of citrus green mold on artificially inoculated fruits stored at 9° C and 90 to 95% relative humidity for four weeks.

Treatment	Percentage disease control *			
	Valencia		Shamouti	
	WE [x]	EE [x]	WE [x]	EE [x]
1 000 ppm garlic	83 b	50 d	70 c	44 c
3 000 ppm garlic	83 b	50 d	60 d	44 c
5 000 ppm garlic	83 b	65 c	75 c	26 e
1 000 ppm garlic + oil	100 a	93 a	85 b	44 c
3 000 ppm garlic + oil	100 a	85 b	60 d	50 c
5 000 ppm garlic + oil	100 a	65 c	50 d	39 d
Oil alone (0.1% v/v)	67 c	65 c	23 e	22 e
1 000 ppm garlic + wax	67 c	61 c	65 c	60 b
3 000 ppm garlic + wax	55 d	56 d	55 d	58 b
5 000 ppm garlic + wax	83 b	80 b	60 d	57 b
Wax alone	55 d	55 d	60 d	62 b
20% ethanol	-	25 e	-	37 d
Water control	17 e	-	15 e	-
Fungicide [y]	100 a	-	100 a	-

[x] WE represent water extracts; EE represent ethanol extracts; [y] Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}).

* Mean of three replicates. Means followed by the same letter in the same column are not significantly different according to Fishers' protected t-test (P = 0.05).

Table 6.6 Effects of garlic extracts on their own, or in combination with vegetable oil, or fruit wax, on control of citrus blue mold on artificially inoculated fruits stored at 9° C and 90 to 95% relative humidity for four weeks.

Treatment	Percentage disease control *			
	Valencia		Shamouti	
	WE [x]	EE [x]	WE [x]	EE [x]
1 000 ppm garlic	80 c	72 c	70 c	76 c
3 000 ppm garlic	91 b	87b	84 b	81 b
5 000 ppm garlic	92 b	83 b	80 b	82 b
1 000 ppm garlic + oil	91 b	77 c	89 b	80 b
3 000 ppm garlic + oil	100 a	85 b	100 a	82 b
5 000 ppm garlic + oil	100 a	85 b	100 a	80 b
Oil alone	52 e	46 e	44 e	52 e
1 000 ppm garlic + wax	71 d	65 d	69 c	60 d
3 000 ppm garlic + wax	88 b	82 b	88 b	70 c
5 000 ppm garlic + wax	92 b	84 b	85 b	81 b
Wax alone	50 e	47 e	52 d	31 f
20% ethanol	-	42 e	-	37 f
Water control	31 e	-	27 f	-
Fungicide	100 a	-	100 a	-

[x] WE represent water extracts; EE represent ethanol extracts; [y] Fungicide represent Fungazil (imazalil 75% a.i. at 1g L^{-1}) plus Decotine (quazatine 20% a.i. at 1 ml L^{-1}

* Mean of three replicates. Means followed by the same letter in the same column are not significantly different according to Fishers' protected t-test (P = 0.05).

Effect of treatment on pathogen development on fruit

Results obtained were similar to the *in vitro* trials and data is therefore not presented. Pathogen growth on plates seeded with spores harvested from treated fruits was much slower compared to the control i.e. fruits treated with only water. Growth rate was not significantly dependent on concentration of extract, and there was no significant difference between spores harvested after 6 and 12 hours of treatment. There was also no difference in growth between spores harvested from Valencia or Shamouti. None of the treatments completely inhibited pathogen growth after six days of incubation at 25°C. The growth of spores harvested from treatment combinations with oil was however, much smaller (not more than 7 mm), and unlike the control, no sporulation of the fungus was observed on these treatments.

6.4.2.1.2. *Coprosma repens*

All concentrations of *C. repens* were effective when compared with the water check in the control of all three pathogens screened. Extracts were more effective in controlling green mold than blue mold. The percentage disease control varied between 80 to 82%, and 64 to 71% in terms of green- and blue mold respectively (Table 6.7). Unlike the *in vitro* study, results of the *in vivo* evaluation showed no significant difference in the percentage disease control obtained at 5 000, 10 000 and 20 000 ppm.

Effect of storage duration and storage temperature on efficacy of extracts

Both storage temperature and storage time (up to 90 days) had little effect on efficacy of extracts. Results presented in Table 6.8 indicated that a lower incidence of blue mold was recorded at all time intervals and temperatures relative to green mold. No remarkable difference in percentage disease control was recorded at 30, 60, and 90 days following storage at both temperature regimes. The percentage control under cold storage varied between 85 and 64% in *Penicillium* and 91 and 87% for *G. citricarpa.*

Table 6.7 Efficacy of ethanol extracts of *Coprosma repens* for the control of *Penicillium digitatum*, *Penicillium italicum*, and *Guignardia citricarpa* on fruits stored at 9° C and 90 to 95% relative humidity for four weeks (in respect of *P. digitatum* and *P. italicum*), and 7° C for three weeks followed by 23° C for one week (in the case of *G. citricarpa*).

Concentration of extract ('000 ppm)	Percentage disease control *		
	Pd [x]	Pi [y]	Pc [z]
20	82 a	71 a	95 a
10	80 a	67 ab	88 ab
5	80 a	64b	83 b
0 (control)	5 b	10 c	13c

[x] represent *P. digitatum*; [y] represent *P. italicum*; [z] represent *G. citricarpa*

* Mean of three replicates and three repetitions. Means followed by same letter in the same column are not statistically significant according to Fishers' protected t-test (P = 0.05).

Table 6.8 Effects of storage duration and temperature on the *in vivo* efficacy of *Coprosma repens* extracts for control of *Penicillium digitatum*, *Penicillium italicum*, and *Guignardia citricarpa*

Concentration of extract ('000 ppm)	Storage temperature/Percentage disease control *					
	23±1° C			9° C		
	Pd [x]	Pi [y]	Gc [z]	Pd [x]	Pi [y]	Gc [z]
After 30 days						
20	72 a	68 a	-	82 a	71 a	90 a
10	70 a	69 a	-	80 a	67 ab	88 a
5	70 a	67 a	-	80 a	64 b	88 a
0 (control)	1 a	3 b	-	5 b	10 c	10 b
After 60 days						
20	73 a	70 a	-	85 a	75 a	91 a
10	71 a	70 a	-	82 a	74 a	87 a
5	71 a	68 a	-	83 a	71 ab	88 a
0 (control)	3 b	5 b	-	3 b	6 c	15 c
After 90 days						
20	75 a	69 a	-	80 a	77 a	89 a
10	71 ab	70 a	-	78 a	70 a	89 a
5	70 ab	68 a	-	80 a	69 a	88 a
0 (water control)	8 b	5 b	-	8 b	3 b	11 c

[x] represent *P. digitatum*; [y] represent *P. italicum*; [z] represent *G. citricarpa*.

* Mean of three replicates and three repetitions. Means followed by same letter in the same column are not statistically significant according to Fishers' protected t-test (P = 0.05).

6.5. Discussion

The increasing reports of pathogen resistance to many conventional fungicides is partly the result of a continuous and often indiscriminate use of the same compound(s) on the same organisms for long periods of time. Part of the solution to this problem is the use of an integrated approach, using different methods/means of control either together or in sequence (Janisiewicz and Korsten, 2002). It is in the light of this new approach to disease management that the results obtained in this study are useful as an alternative means of control of these three citrus pathogens.

Results obtained in the present study indicated that garlic extracts are effective in the control of *P. digitatum, P. italicum,* and *G. citricarpa*. This finding is in agreement with earlier reports (Russell and Mussa, 1977; Garcia and Garcia, 1990; Obagwu *et al.*, 1997) amongst others, on the antifungal properties of garlic clove extracts. This is, however, the first report where garlic clove extracts were evaluated in combination with vegetable oil and plain fruit wax to assess possible improvement in biological activity. The efficacy of extracts, especially in the inhibition of *P. digitatum* and *P. italicum* was positively related to both extract concentration and duration of spore suspension in extracts. Extracts were more effective in inhibition of G. *citricarpa* than *Penicillium* species. This observation is probably due to the fact that *G. citricarpa* is a much slower growing fungus than the *Penicillium* species. Results obtained in this study indicate that garlic extracts had a negative effect on pathogen spore germination, with percentage inhibition positively related to extract concentration.

No growth of *P. digitatum* or *P. italicum* was recorded on treatments comprising 7 000 ppm garlic and above. Results obtained however, indicate that some spores did germinate at these concentrations. This shows that in addition to inhibiting spore germination, extracts also have negative influences on mycelial development; hence, although some spores did germinate at these concentrations, the residual activity of extracts inhibited further mycelial development and so no visible growth was recorded on media. *In vivo* bioassays indicated that extracts were more effective in inhibiting *P. italicum* than *P. digitatum*. This was contrary to observations made in *in vitro* studies. Both pathogens require similar growth conditions. However, under the same environmental conditions, *P. digitatum* grows faster than *P. italicum* (Carlos, 1982). This slower growth rate under

similar conditions could explain the lower growth and/or disease incidence observed in most instances.

A remarkable improvement in biological activity was observed when garlic extracts were mixed with oil. As a result, the treatment comprising 1 000 ppm combined with oil was as effective as the fungicide treatment in the control of both green- and blue mold on Valencia oranges. Our observation also showed that, unlike spores recovered from fruits treated with extracts alone, less than 14% of spores recovered from fruits treated with extracts mixed with oil germinated. None of these spores was capable of further growth when plated on PDA, thus indicating that the treatment affected both spore germination and further pathogen development. Allicin, the main biologically active component of garlic clove is reported to lose its beneficial properties quickly as it breaks down and evaporates (Anon, htp/www/3mistral.co.uk/garlic/allicin). Addition of oil to extracts could therefore have provided a light coating that helped to slow down the evaporation process, prevent oxidation and increased the retention time of allicin on the fruit and thus the contact period with spores.

Results obtained shows that a more effective inhibition was recorded when spores were suspended in extracts for a longer period i.e. three-hour was better than one. Allicin is soluble in alcohol and only slightly soluble in water (North and Quadrini, 2000). Our study however, showed that ethanol extracts were not as effective as water extracts. Because ethanol is a volatile compound, it could enhance the volatilization and break down of allicin in ethanol extracts faster than would occur in water extracts. Consequently, though the percentage allicin in ethanol extracts may have been higher than in aqueous extracts immediately following extraction, the percentage lost was equally higher and thus the efficacy and subsequent control much lower. Also, the main antimicrobial effect of allicin is due to its chemical reaction (blockage) of the thio groups of various enzymes, including alcohol dehydrogenase (ADH), thioredoxin reductase, and RNA polymerase required for the pathogenesis of many microorganisms (Ankri and Miralman, 1999). A study by Massantini *et al.* (2000) indicated that ethanol greatly stimulated ADH activity. This means that the activity of ADH in garlic extracted in ethanol would be equally stimulated, resulting in an abundance of ADH in the extract. This development may result in the blockage or impairment of allicin activity in ethanol extracts thus resulting in reduced efficacy of allicin. All these are possible factors that could have contributed to the

reduced efficacy of ethanol extracts relative to the water extracts of garlic recorded in this study.

Results obtained in the present study shows that *C. repens* possesses antifungal properties, and has great potential for control of the three pathogens screened in this study. The possible role of phenolic compounds in plant resistance to diseases has been reported (Harborne, 1980; Ahmed and Beg, 2001, Moure *et al.*, 2001). This is however, the first report where extracts from *C. repens* was evaluated for control of these pathogens in the post harvest arena. HPLC results indicate that hydroxycinamic acid derivatives are the main component of the phenolic compound pool. The involvement of the hydroxycinamic derivatives in plant pathogen resistance/disease control has been reported (Lattanzio *et al.*, 1994; Agioni *et al.*, 1998; Ejechi *et al.*, 1999; Uzi *et al.*, 1999). They were reported to have antifungal activity against several plant pathogens including *P. digitatum* (Lattanzio *et al.*, 1994). Since this family of compounds is the most abundant, it is most likely that they are largely responsible for the activity of the extract. The TLC bioautographic results showed that all concentrations of free acid extracts were inhibitory and prevented germination of all three pathogens. Both ferrulic and *p*-coumaric acids which were found to be the main phenolics in the extracts are believed to be important components of the cell wall where they play an important role in cell wall lignification and ultimately plant resistance to pathogens (McDougall, 1993; Regnier, 1994).

From the results obtained, we can deduce that that extracts of *C. repens* were effective in the control of all three pathogens screened, but a more effective control of *G. citricarpa* was achieved relative to *P. digitatum* and *P. italicum*. Unlike the results obtained for *Penicillium* control, efficacy of extracts did not appear to depend on extract concentration and duration of spore suspension in extracts. The compound identified as the active constituent of *C. repens* i.e. phenolics is more stable than allicin and may have contributed to the effective control recorded. Phenolics have been implicated in the activity of many plant extracts (Matheron, 2001), mainly by the inhibition of spore germination and possibly mycelial development.

Perhaps the most interesting observation with *C. repens* extracts in this study was its activity against *G. citricarpa*. Infection by this pathogen takes place at the immature stages of fruit development and post harvest control has thus far proved difficult. The level

of control in this study was high (over 90% for CBS) and consistent. These results are very promising and the use of this extract in pre-harvest applications, might give total control of *G. citricarpa*. Temperature did not seem to affect extract activity. This characteristic is of importance especially if extracts are used under export conditions where fruits are stored under cold storage for extended periods of time. We advocate that further studies be undertaken to concentrate the extracts so that it could be used in smaller quantities, and as a spray treatment, preferably as an emulsion to enhance the retention of the active compound on the fruit surface. Before the use of these extracts can be recommended however, it has to be registered, in which case it has to pass toxicology tests and other requirements that accompany product registration.

A detailed cost-benefit analysis was not carried out in this study to determine the economic benefits of garlic-oil treatments. Results obtained however showED that over 500 fruits can be treated with a litre of garlic-oil mixture. The average cost of 400g fresh garlic in South Africa at the time of this report was less than 1 US dollar and the cost of 750 ml vegetable oil about 70 cents. This treatment should therefore be economical. Garlic is edible and has not been reported to have any harmful effects. The preparation of extracts is also simple and straightforward. *Coprosma repens* is abundant and the preparation of the extracts is practicable. These technologies should therefore be easy to adopt. Some of the suggestions made with respect to *C.* repens however need further studies. Also, the possibility of using pure allicin rather than aqueous extracts should be exploited. The identification of an acceptable fragrance capable of suppressing the so-called "bad smell" of garlic without interfering with its biological activity needs further research. Observation from the present investigation showed that this smell reduces with time, probably as the allicin concentration drops.

Literature Cited

Agioni, A., Cabras, P.D., Hallewin, G., Pirini, F.M., Remiero, F. and Schirra, M. 1998. Synthesis and inhibitory activity of 7-geranoxycoumarin against *Penicillium* species in citrus fruit. Photochemistry 47: 1521-1525.

Ahmad, I. and Beg, A. 2001. Antimicrobial and photochemical studies on 45 Indian medicinal plants against multi-drug resistant human pathogens. Journal of Ethnopharmacology 7: 113-123.

Amadioha, A.C. 1999. Evaluation of some plant extracts against *Colletotrichum lindemuthianum* in cowpea. Archives of Phytopathology and Plant Protection 32: 141-149.

Ankri, S, and Miralman, D. 1999. Antifungal properties of allicin from garlic. Miros Infection 1: 125-129.

Ark, P.A. and Thompson, J.P. 1959. Control of certain diseases of plants with antibiotics from garlic (A*llium sativum* L.). Plant Disease Reporter 43: 276.

Carlos, R. 1982. Manual and atlas of the Penicillia. Elsevier Biomedical Press, Amsterdam.

Ejechi, B.O., Nwafor, O.E. and Okoko, F.J. 1999. Growth inhibition of tomato rot fungi by phenolic acids and essential oils extracts of pepperfruit. Food Research International 32: 395-399.

Gabe Mirkin, M.D. 2001. Allicin in garlic. htp/www/3mistral.co.uk/garlic/allicin

Garcia, R.P. and Garcia, M.I. 1990. Laboratory evaluation of plant extracts for the control of *Aspergillus* growth and aflatoxin production. Proceeding of the Japanese Association of Mycotoxicology. In: Horticultural Abstracts 60: 65-43.

GenStat for Windows 2000. Release 4.2, Fifth Edition, VSN International Ltd, Oxford, UK.

Harborne, J.B. 1980. Plant phenolics, in: Encyclopaedia of plant Physiology. New series. Bell, F.A. and Chartwood, B.V. (Eds) Vol. 8: pp 329-402. Springer Verlag.

Hostettmann, K. 1997. Strategy For the biological and chemical evaluation of plant extracts. Invited lecture presented at the International Conference on Biodiversity and Resources: Conservation and Utilization, 22-27 November, 1997, Phuket, Thailand.

Janisiewicz, W.J. and Korsten, L. 2002. Biological control of postharvest diseases of fruits. Annual Review of Phytopathology 40: 411-441.

Lattanzio, V., De-Cicco, V., Di-Venere, D., Lima, G. and Salerno, M. 1994. Actifungal activity of phenolics against fungi commonly encountered during storage. Italian Journal of Food Science 6: 23-30.

Massantini, R., Gobattoni, E., Botondi, R. and Mencarelli, F. 2000. Role of alcohol dehydrogenase on ripening of ethanol-treated tomato and kiwifruit. Acta Horticulturae 379: 297-304.

Matheron, M. 2001. Modes of action for plant disease management chemistry. Paper presented at the 11th Annual Desert Vegetable Crop Workshop, December 6 2001, Yuma AZ.

McDougall, 1993. Phenolic cross-links in growth and development of plants. Pages 119-136 in: Scalbent, A (Ed). Polyphenolic phenomena. INNA Eds,, Paris.

Moure, A., Cruz, J.M., Franco, D., Dominguez, J.M., Sineiro, J., Dominguez, H., Nunez, M.J. and Jarajo, J.C. 2001. Natural antioxidants from residual sources. Food Chemistry 72: 145-171.

North, C. and Quadrini, F. 2000. Allicin-the smell of health. htpwww.chem.ok.ac.uk/moom/allicin.htm

Obagwu, J., Emechebe, A.M. and Adeoti, A.A. 1997. Effect of extracts of garlic (*Allium sativum* L.) bulb and neem (*Azadirachta indica* Juss) seed on the mycelial growth and sporulation of *Colletotrichum capsici*. Journal of Agricultural Technology 5: 51-55.

Pordesimo, A.N. and Ilag, I.I. 1976. Toxicity of garlic juice to plant pathogenic organisms. Philippines Journal of Biology 5: 251-258.

Regnier, T. 1994. Les composes phenoliques blé dur: variations au cours du developpement et de la maturation du grain, relations avec e' apparition du la moucheture. Ph.D. Thesis, Universite' Montpellier II, France.

Russell, P.E. and Mussa, A.E. 1977. The use of garlic to control foot rot of *Phaseolus vulgaris* caused by *Fusarium solani f.sp phaseoli.* Annals of Applied Biology 86: 369-372.

Shashikanth, K.N., Basappa, S.C. and Murthy, V.S. 1981. Studies on antimicrobial and stimulatory factors of garlic. Journal of Food Science and Technology 18: 147-148.

Sinha, P. and Saxena, S.K. 1999. Inhibition of fruit rot fungus and fruit fly by leaf extracts of onion and garlic. Indian Journal of Agricultural Sciences 69: 651-653.

Uzi, A., Jameta, O., Shmuel, C., Victor, R. and Moshe, B.J. 1999. Umbelliferone, a phytoalexin associated with resistance of immature Marsh grapefruit to *Penicillium digitatum*. Phytochemistry 50: 1129-1132.

CHAPTER SEVEN

CONTROLLING THE ESTABLISHMENT OF FOOD-BORNE PATHOGENS WITH *COPROSMA REPENS* EXTRACTS

7.1. Abstract

Ethanol extracts of *Coprosma repens* were evaluated *in vitro* for their biological activity against four food-borne bacterial pathogens; *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* 0157-H7, and *Vibrio cholerae*, and *in vivo* against the establishment of these pathogens on orange fruit surfaces. Extracts were evaluated both as a protective and curative treatment. All concentrations of extracts were effective in checking the establishment of the pathogens both *in vitro* and *in vivo* when compared with the control. Efficacy of extracts was dependent on concentration and varied between pathogens. Extracts were more effective when used as a protective treatment, and gave more effective control of *S. typhimurium* and *V. cholerae* than *S. aureus* and *E. coli*. All concentrations above 5000 ppm completely stopped the establishment of *S. typhimurium* and *V. cholerae* both *in vitro* and *in vivo*.

7.2. Introduction

Reported outbreaks of food-borne illnesses involving fruits and vegetables have increased during the last decade (Drapeau and Solomon, 1998; Schlundt, 2002; Tauxe, 2002). A broad spectrum of microbial pathogens can contaminate human food and water supplies, and cause illness after the organism or their toxins are consumed (Tauxe, 2002). The primary cause of food-borne illnesses include mainly bacteria such as *Escherichia coli* 0157-H7, *Salmonella typhimurium*, *Staphylococcus aureus*, and *Vibrio cholerae,* and other organisms which includes *Clostridium*, *Cyclospora* and *Cryptosporidia* (Evers, 1998). Raw fruits and vegetables grow in a natural environment and therefore carry a wide variety of both beneficial and harmful microorganisms (Evers, 1998). In the case of food poisoning due to microorganisms, the food may serve either as an active vehicle in which multiplication occurs, or as a passive vehicle in which no growth takes place (Varman and Evans, 1991). Accurate records of food-borne illnesses are difficult to come by. In the

United States of America, it has been estimated that 76 million cases of food-borne diseases may occur each year resulting in 325, 000 hospitalizations (Schlundt, 2002). The problem is more severe in developing countries (Kaferstein and Abdussalam, 1999).

Since 1977, new or newly characterized food-borne pathogens have been recognized at the rate of approximately one every two years (Tauxe, 2002). This includes microbial contaminants on uncooked fruits and vegetables, and the identification of microorganisms not previously known to be food-borne pathogens. There is also an increase in the emergence of bacterial strains that are resistant to antibiotics (Conko, 1998). This development has been attributed partly to the widespread use of antimicrobials in hospitals and the community (Schlundt, 2002).

The increasing reports of food-borne illnesses originating from the consumption of fruits and vegetables have resulted in a strict legislation being placed on import of fresh fruits and vegetables into most European countries and the United States. This legislation requires that foods imported into these countries be certified within a food safety framework using systems such as Good Agricultural Practice (GAP) and Hazard Analysis Critical Control Point (HACCP). Another area where more restrictive legislation has been put in place pertains to the maximum residue levels (MRL) allowed on foods including fresh fruits and vegetables. Currently, there are no effective methods available to kill food-borne bacteria on fruits and vegetables. A number of "generally regarded as safe" chemicals have been reported to possess bactericidal activity against food-borne pathogens including *Escherichia coli* 0157:H7, *Listeria monocytogenes*, and *Salmonella enteritidis* for example (Friedman *et al.*, 2002). However, none of these chemicals can singly significantly reduce populations of bacterial pathogens. Food-borne pathogens have been managed mainly with disinfectants such as chlorine. Unfortunately, these products have no residual activity and the possibility of re-contamination in treated consignments destined for export is high. Ideally, any product that will provide effective control of food-borne pathogens should therefore possess some degree of residual properties.

Plants contain thousands of constituents and are a valuable source of new and biologically active molecules. Plant extracts including garlic have been reported to be effective in checking the establishment of food-borne pathogens (Briozzo *et al.*, 1998). However, there is still a lack of knowledge on the potential of other plant extracts in this respect. In a

previous trial (Chapter Six), extracts of *Coprosma repens* Hook F. were effective in controlling *Penicillium digitatum* Sacc., *P. italicum* Wehmer, and *Guignardia citricarpa* Kiely. The aim of this study was therefore to screen *C. repens extracts* for any bactericidal activity against *S. typhimurium*, *S. aureus*, *E. coli* 0157-H7, and *V. cholerae.* This is with a view to using the extracts to provide additional control of these food-borne pathogens on citrus fruit surfaces, in addition to controlling the fungal pathogens listed above. These pathogens were chosen based on their frequency of occurrence and the severity of the illnesses that often result from consuming products contaminated by them.

7.3. Materials and Methods

7.3.1. Food-borne-pathogens

Pathogenic isolates of *E. coli* 015-H7, *S. typhimurium*, *S. aureus* and *V. cholerae* were used in this study. The cultures were received from Wilma du Plooy of the Food Safety Laboratory, Plant Pathology Laboratories, University of Pretoria, and maintained at 7±1° C in the media listed in Appendix 4-7.

7.3.1.1. Preparation of pathogen inoculum

In nature, the population i.e. colony forming units (CFU) of food-borne pathogens on fruits and vegetables vary considerably depending on the type of plant and environmental conditions under which the plant is grown or found (Thunberg *et al.*, 2002). The initial inoculum load i.e. CFU used in this investigation varied between 100 to 150. This concentration is rather high but was chosen to test the efficacy of the extracts under extreme inoculum pressure. To obtain this concentration, a serial dilution was prepared from fresh (24-hour) old cultures of pathogens and plated on *Vibrio* Diagnostic Agar (in the case of *V. cholerae*), Violet-Red-Bile-Mug Agar (in the case of *E. coli*), MacConkey agar (in the case of *S. typhimurium)* and Standard 1 Nutrient Agar (in the case of *S. aureus*). See Appendix 4-7 for composition of media. Colony forming units were counted after 24 hours of incubation at 27±1° C. The dilution yielding a population of between 100-150 CFU was used in further evaluations.

7.3.2. Extracts

The extract used in this investigation was prepared as described previously (Chapter Six, 6.3.3.2).

7.3.3. *In vitro* Screening

Two hundred and fifty microlitres of different concentrations of extracts prepared as described earlier (6.3.3.2) was dispensed separately in microtiter plate (Nunc; AEC-Amersham (Pty) Ltd), wells and inoculated with pathogen cells prepared as described in 7.3.1.1. Sufficient cell suspension was added to give a final concentration of 100-150 CFU ml^{-1}. Such a volume was always predetermined in preliminary trials before the actual inoculation in the microtiter plate. The plate was incubated for between 45 minutes to one hour following which 50 µl of extract-cell suspension was pipetted onto 90-mm Petridishes containing 25 ml aliquots of the different media listed previously depending on the bacteria in question. The suspension was evenly spread using a sterile, plastic Beijerinck rod. Plates were incubated at 27±1° C for 24 hours and the CFU on each plate counted. There were five Petridish/replicates per treatment and each treatment was repeated twice. The control consisted of cell suspended in quarter-strength Ringers solution (Merck). Where growth was observed following treatment, the colonies were compared with the original isolate to confirm their identity. Treatments/concentrations that gave promising results in *in vitro* trials were further evaluated *in vivo* (on fruits).

7.3.4. *In vivo* Screening

The biological activity of extracts was evaluated on Valencia orange. Fresh, healthy fruits were surfaced sterilized with 90% ethanol for one minute and air-dried. Squares, approximately 2 cm^2 were drawn on the fruit surface with a waterproof pen to make a checkerboard pattern. Each square represented a replicate. Extracts were evaluated for both protective and curative activity. In screening extracts for protective activities, different concentrations of extracts prepared as described previously (6.3.3.2) were applied on the marked squares using sterile swabs. The extracts were allowed to air dry. Thirty minutes to one hour following application of extracts, treated squares were challenged with food-borne pathogens. This involved dipping a sterile swab in an inoculum suspension

prepared as described in 7.3.1.1, and gently swabbing the squares treated with extracts with the suspension. Three to six hours after challenge-inoculation, swabs were taken from the treated squares using sterile, moist, swabs and streaked out on different media as in 7.3.1.1. Plates were incubated and assessed as described earlier (7.3.3). Controls included squares treated with sterile distilled water only. To ensure that experimental materials were not sources of contamination, plates streaked with swabs only or swabs taken from sterilized but untreated squares were included as controls. To assess the curative properties of extracts, the same procedure described above was followed except that the pathogens were first applied before treatment with extract. Colonies recovered from test trials were always compared with the original cultures to confirm their identity. Where any contamination was observed the experiment was discarded. There were three replicates (squares) per concentration and the experiment was repeated three times.

7.3.4.1. Residual activity of extracts

To assess the residual activity of extracts, fruits treated as described above were stored at 8±1° C for four weeks following which they were evaluated for survival of pathogens as described earlier (7.3.3.1).

7.3.5. Statistical analysis

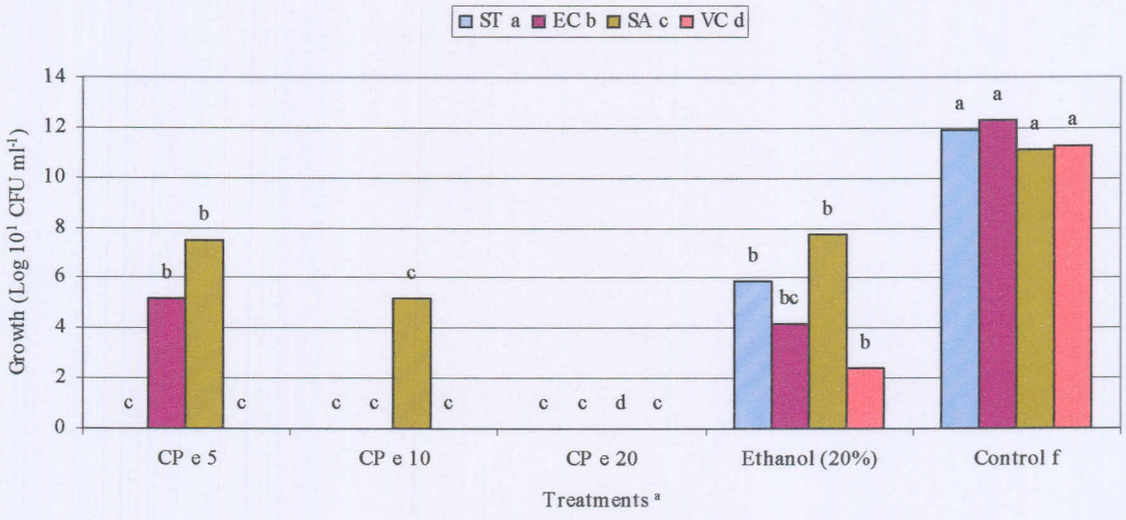
Data was statistically analysed using the GenStat statistical program. One-way analysis of variance (ANOVA) was used to test for differences in average means between treatments. Treatment means were separated using Fishers' protected t-test at a 5% level of significance.

7.4. Results

7.4.1. *In vitro* Screening

Results obtained from the present study indicate that ethanol extracts of *C. repens* possess bactericidal properties. From the results presented in Fig.7.1 it is clear that extracts were effective in checking the growth of *E. coli* 0157-H7, *S. typhimurium*, *S. aureus* and *V. cholerae in vitro* when compared with both the water control, and 20% ethanol on its own. Efficacy of extracts improved with increase in concentration but not in all cases. The

minimum inhibitory concentration (MIC) that completely stopped the growth of *S. typhimurium* and *V. cholerae* for example was 5 000 ppm powder. On the other hand, only concentrations of 10 000 ppm and above completely stopped the growth of *E. coli* 0157-H7 and complete inhibition of *S. aureus*, was achieved only at 20 000 ppm.

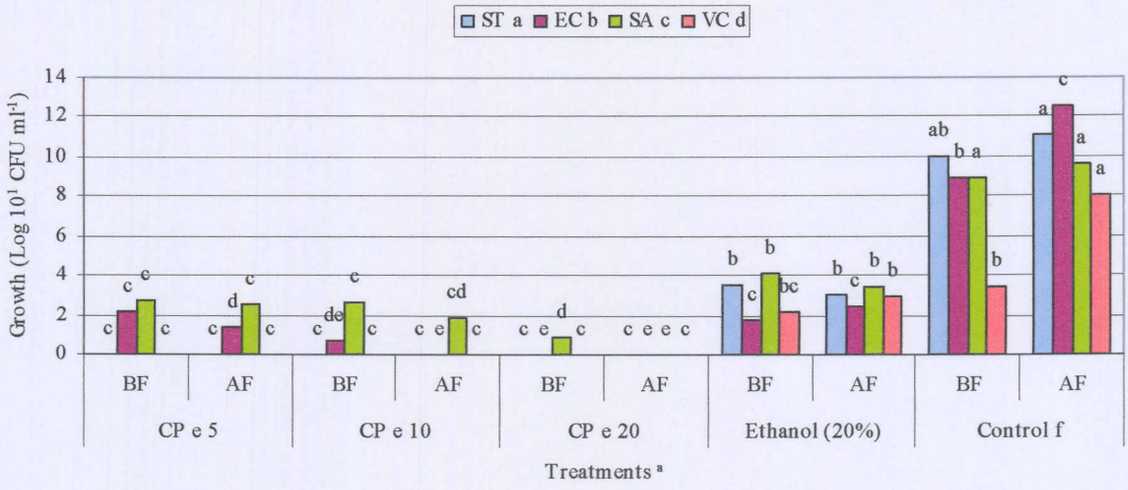


[a] CP 5, 10 and 20 represent concentration of *Coprosma repens* extracts (in 1 000 ppm); Control represents quarter strength Ringer's solution; ST represent *Salmonella typhimurium*; EC represent *Escherichia coli* 0157-H7; SA represents *Staphylococcus aureus*; VC represents *Vibrio cholerae*. Treatments having same letter are not significantly different according to Fishers' protected t-test (P = 0.05).

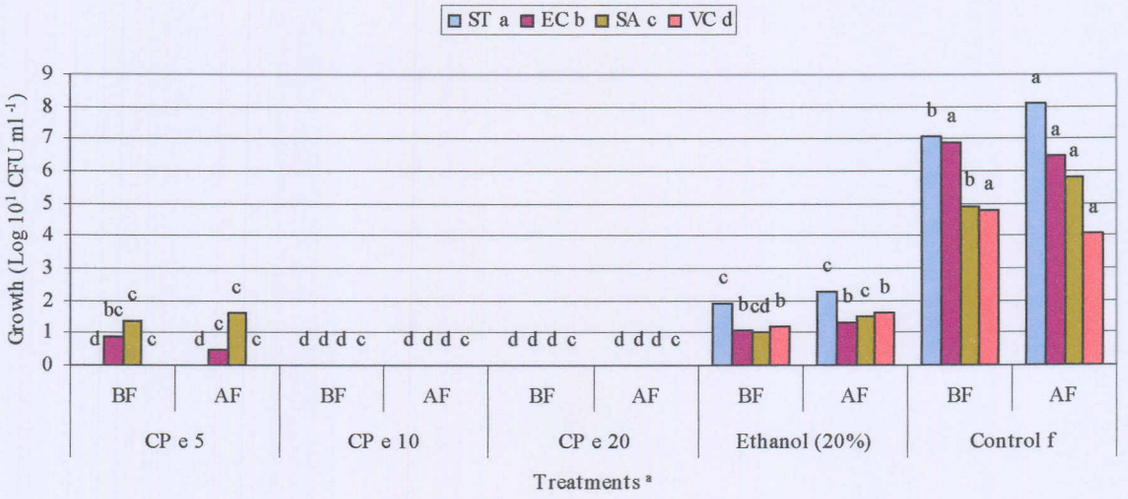
Fig. 7.1 Effect of ethanol extracts of *Coprosma repens* on the population (number of colony forming units) of food-borne bacterial pathogens *in vitro* following incubation at 30° C for 24 hours.

7.4.2. *In vivo* Screening

Results of the *in vivo* study were variable, but extracts were generally more effective in *in vivo* than *in vitro* trials, perhaps because the pathogen population (as determined by CFU) was lower. In general, extracts were more effective when used as a protective rather than a curative treatment. Results presented in Fig. 7.2A shows that the MIC required to completely stop the establishment of both *S. typhimurium* and *V. cholerae* was 5 000 ppm, irrespective of whether it was used as a protective or curative treatment.



A



B

[a] CP 5, 10 and 20 represent concentration of *Coprosma repens* extracts (in 1000 ppm); Control represents quarter strength Ringers solution; ST represent *Salmonella typhimurium*; EC represent *Escherichia coli* 0157-H7; SA represents *Staphylococcus aureus*; VC represents *Vibrio cholerae*; **BF** means fruit is treated with extract before inoculation with pathogen, **AF** means the opposite. Treatments having same letter are not significantly different according to Fishers' protected t-test (P = 0.05)

Fig. 7.2 Effect of ethanol extracts of *Coprosma repens* on the establishment of food-borne bacteria pathogens on Valencia orange fruits surface (A), and the residual effects of extracts on the establishment of pathogens after four weeks of storage at 8±1° C. (B).

Complete control of *E. coli* on the other hand was achieved only at 10 000 ppm, (Fig. 7.2A). Extracts were less effective in the control of *S. aureus*. As a result, only a MIC of 20 000 ppm could completely stop the establishment of the pathogen, and only when the extract was used as a protective treatment.

7.4.3. Residual Activity of Extracts

From the results presented in Fig. 7.2B it is clear that pathogen survival as determined by the CFU was lower after four weeks of cold storage compared with the trials where the extract activity was determined within a few hours after application (7.4.2). As a result, extract performance was higher than the *in vitro* and *in vivo* trials. Unlike the preceding trials however, all concentrations above 10 000 ppm completely inhibited the establishment of all pathogens, irrespective of whether they were used as a protective or curative treatment (Fig. 7.2B).

7.5. Discussion

Results obtained in the present study shows that *C. repens* possess bactericidal properties. All concentrations of extracts were effective when compared with the control (quarter strength Ringer's solution) in checking the establishment of all four food-borne pathogens screened including *S. typhimurium*, *S. aureus*, *E. coli* 0157-H7, and *V. cholerae*. The fungicidal activity of *C. repens* was demonstrated earlier (Chapter Six). In this study, it was found that the compound responsible for extract activity belonged to the hydroxycinamic acids group. Although the antifungal activity of these compounds (including *Penicillium* species.) has been reported (Agioni *et al.*, 1998; Uzi *et al.*, 199), there are no available records of their role as anti-bacterial agents. This is the first report where the anti-bacterial activity of extract of this plant against the four food-borne pathogens screened in this study has been demonstrated.

The efficacy of extracts varied with concentration and between pathogens. Extracts were more effective in checking the establishment of *S. typhimurium* and *V. cholerae* compared to *E. coli* 0157-H7 and *S. aureus*, resulting in complete control of the former pathogens at 5 000 ppm and above. All concentrations of extracts (except 5 000 ppm, and only in the case of *E. coli* 0157-H7) were more effective than the ethanol treatment thus indicating

that ethanol was not solely responsible for extract activity. The exact reason for the lower pathogen population recorded in *in vivo* trials relative to the *in vitro* is not completely understood. The "dry" surface of fruits in cold storage is however not an ideal environment for bacterial growth, most of which require warm and moist environments for optimum growth. Suspending cells of the above pathogens in rind extracts (prepared by homogenizing citrus peel in sterile, distilled water), prior to plating did not affect pathogen growth significantly (data not presented), thus indicating that the lower colony count (population) may not have resulted from any negative effect from the rind on growth.

From the results obtained in the present study, we can deduce that extracts possess some residual activity. After four weeks of storage at 8±1° C, no growth was recorded in all extract concentrations above 5 000 ppm. The same level of control was not recorded with the ethanol treatment and the water control. Although ethanol does have antimicrobial properties, it has a poor residual activity because of its tendency to evaporate quickly. This characteristic reduces its contact period with the pathogen and probably its activity. Results obtained in this study indicated that the activity of extracts was independent of temperature. This property is an advantage over the conventional disinfectant such as chlorine whose activity is easily influenced by changes in temperature. The combined effects of the bactericidal activities of extracts, and low temperature may therefore have resulted in higher cell lysis on fruits kept under cold storage. This observation is particularly interesting as it does indicate that extracts will remain effective even under export conditions where fruits are kept under cold storage conditions for up to four weeks or more.

Results obtained in the present study are also of interest in our effort to control food-borne pathogens especially in the fruit industry where the water used to wash fruit may harbour one or more of the bacteria screened in this study. The effective control achieved with *V. cholerae* is of particular interest since it is the causal agent of cholera disease, an illness that causes many deaths in developing countries. These extracts therefore hold great promise as a potential natural product that could be used as a disinfectant in our quest to reduce food-borne illnesses. There is however, a need for further studies to concentrate the active compound in this extract so that it can be used in smaller volumes as a spray treatment rather than in large volume treatments. Although *C. repens* is not listed as a poisonous plant (Appendix 2), which theoretically makes it safe, there is still the need to

investigate possible side effects of the active component of this extract on man to be sure of its safety when consumed. This will also facilitate its use on other food products. This study however, falls outside the scope of the present investigation.

Literature Cited

Agioni, A., Cabras, C.D., Hallevin, G., Pirini, F.M., Remiero, F. and Schirra, M. 1998. Synthesis and inhibitory activity of 7-gernoxycuramicrin against *Penicillium* species in citrus fruit. Phytochemistry 47: 1521-1525.

Briozzo, J., Nunez, L., Chirife, J., Herszage, L. and D'Aquino, M.. 1998. Antimicrobial activity of clove oil dispersed in a concentrated sugar solution. http//www.bth.co.uk/EssentialOils.htm.

Conko, 1998. On the plate. http:/www.consumeralert.org/pubs/OnthePlate/may1998.htm.

Drapeau, C. and Solomon, N. 1998. Blue-Green Algae: A powerful immune system enchancer.TheExperts'Optimal Health Report.htp/www.galaxymall.com/virtual/shgalgae/research.htm.

Evers, W.D. 1998. Electronic Food RAP. Volume 8 (35). htp/www.cfu.purdue.edu/extension/efr/efrframe.htm.

Friedman, M., Henika, P.R. and Mandrell, R.E. 2002. Bacterial activities of plant essential oils and some of their isolated constituents against *Campylobacter jejuni*, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella enterica*. Journal of Food Protection 65: 1545-1560.

GenStat for Windows 2000. Release 4.2, Fifth Edition, VSN International Ltd, Oxford, UK.

Käferstein, F. and Abdussalam, M. 1992. Food safety in the 21st century. Bulletin of the World Health Organization 77: 347-351.

Schlundt, I. 2002. New directions in food-borne disease prevention. International Journal of Food Microbiology 78: 1-17.

Tauxe, R. 2002. Emerging food-borne pathogens. International Journal of Food Microbiology 78: 31-41.

Thunberh, R.L., Tran, T.T., Bennett, R.W., Matthews, R.R. and Belay, N. 2002. Microbial evaluation of selected fresh produce obtained at retail markets. Journal of Food Protection 65: 677-682.

Uzi, A., Jameta, O., Shmuel, C., Victtor, R. and Moshe, B.J. 1999. Umbelliferone, a phytoalexin associated with resistance of immature grapefruit to *Penicillium digitatum*. Phytochemistry 50: 1129-1132.

Varnam, A.H. and Evans, M.G. 1991. Foodborne pathogens: An illustrated text. Mosby Yearbook Inc. New York.

CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSION

The citrus plant is susceptible to attack by many diseases both pre- and postharvestly. Citrus black spot (CBS) caused by *Guignardia citricarpa* Kiely is responsible for high economic losses annually resulting from the rejection of export consignments to countries regarded as CBS free. The molds caused by *Penicillium* species, particularly *Penicillium digitatum* Sacc. and *P. italicum* Wehmer the cause of citrus green- and blue mold respectively, are of economic importance in all citrus producing regions of the world (Eckert and Eaks, 1989). These diseases are currently managed with pre-harvest fungicides (CBS) or postharvestly, in the case of green- and blue mold. However, growing concern over the residual effects of chemicals in the food-chain, and the subsequent danger posed to human health (Norman, 1988), have resulted in a renewed search for alternative disease control measures. Reports of food-borne illnesses have increased in the last decade (Drapeau and Solomon, 1998; Schlundt, 2002; Tauxe, 2002). Thus far, no control measure has proved effective against food-borne pathogens on fruits. All these aspects are of importance in terms of international trade and therefore a study was initiated focusing on developing new postharvest control options for CBS, green- and blue mold. The study was therefore conducted to evaluate *Bacillus* species, either on their own or in combination with other non-chemical products for their biological activity against the most important trade pathogens. The choice of *Bacillus* was based on some desirable morphological characteristics such as endospore formation, which makes them resistant to adverse environmental conditions including long term cold storage conditions and because of previous successful reports in controlling citrus diseases (Huang *et al.*, 1992; Auret, 2000; Korsten *et al.*, 2000), and reports of previous inhibitory activity. Plant extracts were also screened for possible activity against these pathogens due to renewed interest in this untapped resource. The possibility of using plant extracts to control the establishment of food-borne pathogens on citrus fruits in addition to fungal pathogens was also investigated.

Results obtained in the present study showed that some of the *Bacillus* species screened have excellent antagonistic properties and therefore high potential for use in control of *P. digitatum* and *P. italicum* in the postharvest arena. The isolates on their own were not as

effective as the commercial fungicide treatment (imazalil 1 000 ppm + quazatine 1 000 ppm) that gave total control of both pathogens in all experiments. Three of the *Bacillus subtilis* isolates screened; F1, L2 and L2-5, were very effective in controlling *P. digitatum* and *P. italicum* both *in vitro* and *in vivo* (i.e. on fruit). Of these three, F1 was particularly outstanding in its performance and consistency. A remarkable improvement in biocontrol activity of isolates was observed when they were combined with sodium bicarbonate (SB). When combined with SB, isolate F1 was as effective as the fungicide treatment in the control of both *P. digitatum* and *P. italicum*. The antifungal activity of *Bacillus* species (Singh and Deverall, 1984; Huang *et al.*, 1992; Korsten *et al.*, 2000; Obagwu *et al.*, 2001) and the inhibitory activity of SB (Barger, 1928; Smilanick *et al.*, 1999; Palou *et al.*, 2001) have been reported. This is however, the first report where *B. subtilis* isolates were evaluated together with SB to exploit the potential benefits of such integration. These results are of interest for the postharvest arena in the search for alternative control options. It is generally thought that the main mode of action of *Bacillus* species is antibiotic production. Results obtained in this study however showed that antibiotics were not the main mode of action of the three *B. subtilis* isolates tested. This property should make these isolates more acceptable for use in postharvest treatments. The use of F1 combined with SB for control of *P. digitatum* and *P. italicum* is therefore recommended. However, observations reported herein are only the first step in the commercialization process of a biocontrol product. These isolates therefore need to be further screened for non-target activities, toxicological properties and consistency under commercial conditions.

In the present study, we observed that extracts of garlic (*Allium sativum* L.), clove and *Coprosma repens* Hook F. were effective in the control of *P. digitatum*, *P. italicum* and *G. citricarpa*. Extracts on their own were not as effective as the commercial fungicide treatment. A remarkable improvement in the biocontrol activity of garlic extracts was observed when the extract was combined with vegetable oil. A combination of extracts (1 000 ppm) with vegetable oil (0.1%) for example was as effective as the fungicide treatment which gave complete control of both *P. digitatum* and *P. italicum* on fruits. The antifungal activity of garlic has been reported (Bisht and Kamal, 1994; Obagwu *et al.*, 1997; Sinha and Saxena, 1999). This is however the first report where garlic clove extracts were evaluated together with vegetable oil to improve its biological activity. Ethanol extracts of C. *repens* was effective in both *in vitro* and *in vivo* evaluation in controlling the growth of the three pathogens listed. Of particular interest was the observed activity

against *G. citricarpa* as measured by the inhibition of the development of new CBS lesions. The postharvest control of CBS has proved to be very difficult, and so far, no fungicide is known to provide total control of postharvest symptom development. *Coprosma repens* extracts therefore hold great promise as an alternative control measure for citrus postharvest pathogens and CBS in particular. Since CBS infection is initiated at the immature stages of fruit development, we postulate that a combination of pre-harvest applications, supplemented with postharvest treatments as was done in this study might provide more effective control of this pathogen. The possibility of build up of pathogen resistance should however, be investigated if a pre- and postharvest approach is to be followed. This approach should therefore be evaluated in further studies. The possibility of concentrating the active component of this extract and using it in smaller quantities, as an emulsion spray to improve the retention of the active component on the fruit surface is also advocated, and should be investigated. Another interesting observation with respect to *C. repens* extract was its activity against the establishment of the four food-borne pathogens tested in this investigation i.e. *Salmonella typhimurium*, *Staphylococcus aureus*, *Escherichia coli* 0157-H7, and *Vibrio cholerae*. The effective control of these pathogens with this extract is interesting not only because of the importance of these pathogens, but also because to date, there are still no effective means of controlling these pathogens on fruits.

In conclusion, this study has shown that the combination of isolate F1 of *B. subtilis* with SB was effective and consistent in its performance and therefore has great potential for commercial use in the postharvest arena for control of both *P. digitatum* and *P. italicum* on citrus. The use of this technology for the export market is therefore recommended. It is important to note however, that a commercial formulation of F1 was not tested and no commercial trials were carried out. Equally important is the fact that preliminary results showed that these biocontrol systems were not effective in the control of sour rot. No further testes were therefore carried out or reported. Also recommended is the use of a mixture of garlic clove extracts (1 000 ppm) with vegetable oil (0.1%) for control of *P. digitatum* and *P. italicum* on citrus. Based on the results obtained in this study, ethanol extracts of *C. repens* have great potential for the management of CBS, postharvest *Penicillium* rots, and the establishment of the four important food-borne pathogens tested in this study. We strongly advise that this extract be evaluated further.

Suggestions for future studies:

1. Commercial evaluation of treatment F1 + SB under exact export conditions, monitoring fruits overseas.
2. Evaluate the effects of a combined pre- and postharvest application using extracts of *C. repens* for control of CBS.
3. Evaluate the effects of integrating *C. repens* extracts with other non-chemical control measures (such as SB).
4. Determine the non-target effects of *C. repens*.
5. Evaluate product consistency by repeating commercial trials in different regions, on several cultivars and throughout the season.
6. Conduct further studies to determine factors that affect genetic stability of isolates.

Literature Cited

Auret, E.L. 2000. Control strategies for citrus post harvest diseases. MSc. Thesis, University of Pretoria, Pretoria

Barger, W.R. 1928. Sodium bicarbonate as a citrus disinfectant. California Citrograph 12: 164-174.

Bisht, S.S. and Kamal, G.B. 1994. Garlic extract-an effective antifungal treatment for the control of storage rot of apple. Proceeding of the National Academy of Sciences India 64: 233-234.

Drapeau, C. and Solomon, N. 1998. Blue-Green Algae: A powerful immune system enchancer.The Experts'Optimal Health Report.htp/www.galaxymall.com/virtual/shgalgae/research.htm

Eckert, J.W. and Eaks, J.L. 1989. Postharvest disorders and diseases of citrus fruit. Pages 179-260 in: The citrus industry. Vol. 5. Reuther, W. Calavan, E.E. and Carman, G.E. (Eds). University of California, Division of Agriculture and Natural Resources, Ock.

Huang, Y., Wild, B.L. and Morris, S.C. 1992. Postharvest biological control of *Penicillium digitatum* decay on citrus fruit by *Bacillus pumilus*. Annals of Applied Biology 130: 367-372.

Korsten, L., de Jager, E.S., Paul, I., Obagwu J. and El Ghaouth, A. 2000. Alternative control of citrus postharvest diseases. Poster presented at the International Society of Citriculture at Orlando, Florida, USA, 3-7, December 2000.

Norman, C. 1988. EPA sets new policy on pesticide cancer risks. Science 242: 366-367.

Obagwu, J., Emechebe, A.M. and Adeoti, A.A. 1997. Effect of extracts of garlic (*Allium sativum* L.) bulb and neem (*Azadirachta indica* Juss) seed on the mycelial growth and sporulation of *Colletotrichum capsici*. Journal of Agricultural Technology 5: 51-55.

Obagwu, J., Korsten, L., Wehner, F.C. and Paul, I. 2001. Inhibitory action of natural epiphytes isolated from citrus fruit surface against *Penicillium digitatum* Sacc., the cause of citrus green mold. Paper presented at the 39th South African Society for Plant Pathology Congress. Greenway Woods, Nelspruit, South Africa, 21-24 January 2001.

Palou, L., Smilanick, J.L., Usall, J. and Virias, I. 2001. Control of postharvest blue and green molds of oranges by hot water, sodium carbonate and sodium bicarbonate. Plant Disease 85: 371-376.

Schlundt, I. 2002. New directions in food-borne disease prevention. International Journal of Food Microbiology 78: 1-17.

Singh, V. and Daverall, S.J. 1984. *Bacillus subtilis* as a control agent against fungal pathogens of citrus fruit. Transaction of the British Mycological Society 83: 487-490.

Sinha, P. and Saxena, S.K. 1999. Inhibition of fruit rot fungus and fruit fly by leaf extracts of onion and garlic. Indian Journal of Agricultural Sciences 69: 651-653.

Smilanick, J.L., Margosan, D.A., Mlikota, F., Usall, J. and Michael, F. 1999. Control of citrus green mold by carbonate and bicarbonate salts and the influence of commercial post harvest practices on their efficacy. Plant Disease 83: 139-145.

Tauxe, R. 2002. Emerging food-borne pathogens. International Journal of Food Microbiology 78: 31-41.