CHAPTER 6

PRODUCTION AND PARTIAL CHARACTERISATION OF INHIBITORY METABOLITES PRODUCED BY *BACILLUS SUBTILIS*

1. ABSTRACT

The mode of action of *Bacillus subtilis* as biocontrol agent is often ascribed to the production of inhibitory secondary metabolites. Some of these metabolites reportedly have a wide spectrum of antifungal activity. Fungal pathogens causing postharvest avocado diseases are effectively controlled under commercial conditions using a strain of *B. subtilis*. Inhibitory substances produced *in vitro* pointed to antibiosis as one of the modes of action involved in the interaction. The *in vitro* production of inhibitory metabolites by *B. subtilis* was evaluated over time to determine the optimal time for extracting large quantities of the metabolite. A high concentration of inhibitory substances was present in the cell free filtrate of *B. subtilis* growth media by day seven. The possibility that the inhibitory substance may be a phenolic compound was investigated. Antifungal compounds, inhibitory to *Cladosporium cladosporioides*, were found in the free acid fraction of *B. subtilis* growth medium. The fraction was analysed using thin layer chromatography and high performance liquid chromatography and results indicate that the phenolic compounds produced belong to the hydroxycinamic family of compounds.

2. INTRODUCTION

Using *Bacillus subtilis* as a biocontrol agent against fungal plant pathogens has been investigated by numerous researchers (Loeffler *et al.*, 1986; Pusey *et al.*, 1986; Kugler *et al.*, 1990; Fiddaman & Rossall, 1993; Korsten *et al.*, 1993; Ikediugwo *et al.*, 1994; Podile & Prakash, 1996; Sailaja *et al.*, 1997). Antibiosis is commonly employed in biocontrol by *B. subtilis* (McKeen *et al.*, 1986; Gueldner *et al.*, 1988; Leifert *et al.*, 1995). In previous studies, *B. subtilis* proved to be an effective biocontrol agent against avocado postharvest diseases (Korsten *et al.*, 1991; Korsten, 1993). A commercial product, Avogreen®, containing *B. subtilis*, was formulated and registered (Korsten *et al.*, 1998). Studies on the mode of action involved in the antagonism of *B. subtilis* against postharvest fungal pathogens of avocado provide evidence that one or more antifungal metabolites are involved (Korsten & de Jager, 1995; Havenga *et al.*, 1999).

The production of antibiotic metabolites by the specie *B. subtilis* is well documented (Chapter 2) and it is also common to find more than one antibiotic metabolite produced. Cell-free filtrates of *B. subtilis* were reportedly

used to protect fruit from *Monilinia fructicola* (Wint.) Honey (McKeen *et al.*, 1986). The inhibitory substances were isolated and several iturin peptides were identified, active against a wide variety of fungi (Gueldner *et al.*, 1988). Most research has focused on peptide and lipopeptide antibiotics (Katz & Demain, 1977; Shoji, 1978; Peypoux *et al.*, 1984; Loeffler *et al.*, 1986; Sakajoh *et al.*, 1987; Jacques *et al.*, 1994; Chen *et al.*, 1995; Klein *et al.*, 1996; Tsuge *et al.*, 1996; Lin *et al.*, 1998; Pinchuk *et al.*, 2002). Less information is available concerning non-peptide antibiotics, including phenolic compounds (Pinchuk *et al.*, 2002).

The aim of this study was to determine what inhibitory substances are produced by *B. subtilis* and if phenolic compounds are partly responsible for antagonism.

3. MATERIALS AND METHODS

3.1. Strains

Bacillus subtilis subculture MI-14 (Chapter 3) was used throughout this study. The antagonist was stored in 30 % glycerol with Ringer's (Merck, Johannesburg, S.A.) solution at –70 ºC. Cultures were maintained on standard 1 nutrient agar (STD1) (Biolab, Merck) and plates were incubated at 28 ºC for 24 h before use.

Colletotrichum gloeosporioides Penzig. (Chapter 3) and *Cladosporium cladosporioides* (Fresen.) G.A. de Vries (M. Muller, Department of Microbiology and Plant Pathology, University of Pretoria) was maintained on potato dextrose agar (PDA) (Biolab, Merck) slants as well as in sterile water at room temperature. An agar disk containing mycelia and spores of the fungus was placed on PDA and incubated at room temperature for three days prior to use. Fungal spore suspensions were made by pipetting sterile water onto PDA plates and removing mycelia and spores with a sterile streaking rod (Chapter 4).

All solvents used in this study were supplied by uniLAB (Saarchem, Merck, Johannesburg, S.A.).

3.2. Optimising antifungal metabolite production by Bacillus subtilis

Antibiotic production medium (APM) (McKeen *et al.*, 1986) was inoculated with *B. subtilis*. Antibiotic production media contained the following: 2 % (m/w) D-(+)-glucose and 0.5 % (m/w) DL-glutamic acid (both from Sigma, Johannesburg, S.A.); as well as 4.13 mM $MgSO_4.7H_2O$; 5.74 mM K_2HPO_4; 6.7 mM KCl (all from Saarchem, Merck); and 1 ml trace element solution (2.95 mM $MnSO_4.H_2O$ (Saarchem); 0.64 mM $CuSO_4.5H_2O$ (Pro Analysi, Merck); 0.05 mM $FeSO_4.7H_2O$ (Sigma) in 100 ml distilled water) per litre distilled

water. The pH was adjusted to 6.0 – 6.2. Erlenmeyer flasks (250 ml) containing 100 ml APM were autoclaved at 121 ºC for 15 min. Flasks were inoculated with a single colony of bacteria growing on STD1 and shake incubated (70 rpm) at 25 ºC for seven days. Absorbency measurements were taken **(240 nm)** and viable counts were made daily for seven days. In addition, two ml of the culture suspension was filtered through a 0.22 µm pore sized acetate filter (Millipore, Separation Scientific, Johannesburg). Spore suspensions were made as described above and were spread plated over PDA. Holes were made in the agar with a five mm diameter cork borer and 0.25 ml of the filtrate was pipetted into the hole. Three holes were made per Petridish, serving as one replicate. The absence or presence of inhibition zones were noted and were measured when formed. A filtrate of sterile antibiotic production medium was used as a negative control. The experiment was done in duplicate. Data was statistically analysed using Microsoft® Excel 2000.

3.3. Antifungal metabolite production and partial characterisation

3.3.1. Antifungal metabolite production

Flasks containing sterile APM were inoculated with a single colony of bacteria growing on STD1 and shake incubated (70 rpm) at 25 ºC for seven days.

3.3.2. Extraction of antifungal metabolites

On day seven, production medium was centrifuged (14 000 x g) for 10 min to remove bacterial cells. The supernatant was filtered through a 0.22 µm pore sized acetate filter into Eppendorf tubes (crude cell-free extract) and stored in the dark at 4 ºC until needed.

3.3.3. Extraction of free acid phenolic compounds

The pH of the crude cell-free extract was lowered to ± 2.6 by adding tetrafluroacetic acid (TFA) at a final concentration of 20 % (v/v) and mixed well. A 1:1 (v:v) solution was made with diethylether. The solution was mixed and allowed to stand for five minutes to allow separation of the different phases. The supernatant was removed with a micropipette and placed in a new Eppendorf tube. This procedure was repeated four times. The supernatants were combined and diethyl ether allowed to evaporate completely. The resulting precipitate was dissolved in 100 µl methanol and stored at 4 °C. An extraction of sterile APM was also done and served as the negative control.

3.3.4. Quantification of total phenolic compounds

Total soluble phenolics were quantified using the Folin-Ciocalteu's reaction (Swain & Hillis, 1959; Harborne, 1984). The volumes used were scaled down in order to use 96-well microtiter plates (Lasec, Johannesburg). Microtiter plate wells were inoculated with 370 µl distilled water. Samples to be analysed were added to the well at 5 µl per well, followed by 50 µl of 20% (w/v) Na_2CO_3 (Sigma) solution. Folin Ciocalteu's Phenol reagent (Sigma) was added as a colorimetric indicator to each well at 25 µl per well. Water served as negative control. The solutions in each well were mixed thoroughly with a micropipette and the plate was incubated at 37 °C for 30 minutes. Three wells were used per sample and the analysis was done in triplicate. Absorbency was measured with a Multiskan Ascent VI. 24 354-00973 (version 1.3.1). Data were calculated as equivalent gallic acid in mg / ml extract from the standard curve using an equation: $y = 1.3527x + 0.0109$ ($R^2 = 0.9989$) (Dr. T. Regnier, 2000, personal communication).

3.3.5. Separation of free acid phenolic compounds by thin layer chromatography

Thin layer chromatography (TLC) was used to evaluate possible antifungal compounds produced by *B. subtilis*. Crude extract, consisting of cell-free filtrates of *B. subtilis* growth medium at day seven, free acid extract and the growth medium used as control were analysed. For optimal separation of phenolic compounds, various solvents were tested, namely benzene:acetic acid:water (6:7:5, v/v/v) (BAW), toluene:acetic acid (4:1, v/v), acetic acid:methanol:water (8:1:1, v/v/v) (AMW) and water alone. Pre-coated glass plates (Silica Gel 60 F-254) as well as aluminum pre-coated TLC plates (SIL G-100UV_{254}) (both from Merck) were tested and the glass plates proved to be the better basis. All assays were run in triplicate and spots and bands were visualized with a CAMAT 50 Hz UV lamp (254 and 366 nm).

3.3.6. Antifungal activity of separated fluorescent spots against Cladosporium cladosporioides

Three volumes, 10, 20 and 30 µl, of the crude extract as well as the free acid extracts were spotted onto the TLC plates and ran with AMW. The plates were left under an extractor fan overnight to remove all traces of volatile solvents. The method described by Homans & Fuchs (1970) was used to detect antifungal activity. Plates were sprayed with a nutrient broth containing *C. cladosporioides* at 10^6 spores per ml. The broth consisted of two solutions, A and B, mixed together in a ration of 1:6 A:B (v/v). Solution A consisted of a 30 % (w/v) glucose (Sigma) solution. Solution B contained 0.04 M KH_2PO_4 (Saarchem), 0.02 M Na_2HPO_4 (Sigma), 0.04 M KNO_3 (Sigma), 4.1 mM $MgSO_4.7H_2O$ (Saarchem) and 0.02 M NaCl (Saarchem). Plates

were incubated in a moisture chamber at 25 ºC for 48 to 96 h and observed for the formation of clear inhibition zones.

3.3.7. High performance liquid chromatography of free acid phenolic compounds

Dr. T. Regnier (Department of Microbiology and Plant Pathology, University of Pretoria) performed the high performance liquid chromatography (HPLC) analysis. The sample (10 µl) was injected into a HPLC column. The chromatographic system consisted of Varian 9012 high pressure pumps (three phases), a manual injector, an integrated system controller, a MALsil C18, 5 micron, reverse-phase analytical column (250 x 4.6 mm, five µm 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). The mobile phase, consisting of water and acetonitrile, as well as the flow rate used at specific times are summarized in table 6.1.

Table 6.1: The program followed during separation of samples using high performance liquid chromatography

Time: Minutes	% Acetonitrile	Flow rate
0	10	1.5
10	30	1.6
13	50	1.9
15	55	1.9
17	10	1.6

4. RESULTS

4.1. Optimising antifungal metabolite production by Bacillus subtilis

Antifungal substance concentration increased with the cell concentration in the seven day incubation period (Figure 6.1). After day five, the inhibition zone formed started to level off and it was decided that further analysis should focus on the antifungal metabolites present on day seven.

4.2. Quantification of total phenolic compounds

Phenolic compounds were present in the free acid extract at a concentration equivalent to 7.06 ± 0.95 mg gallic acid ml^{-1}. The growth medium was used as control and contained a total of phenolic compounds equivalent to 0.04 ± 0.01 mg gallic acid ml^{-1}.

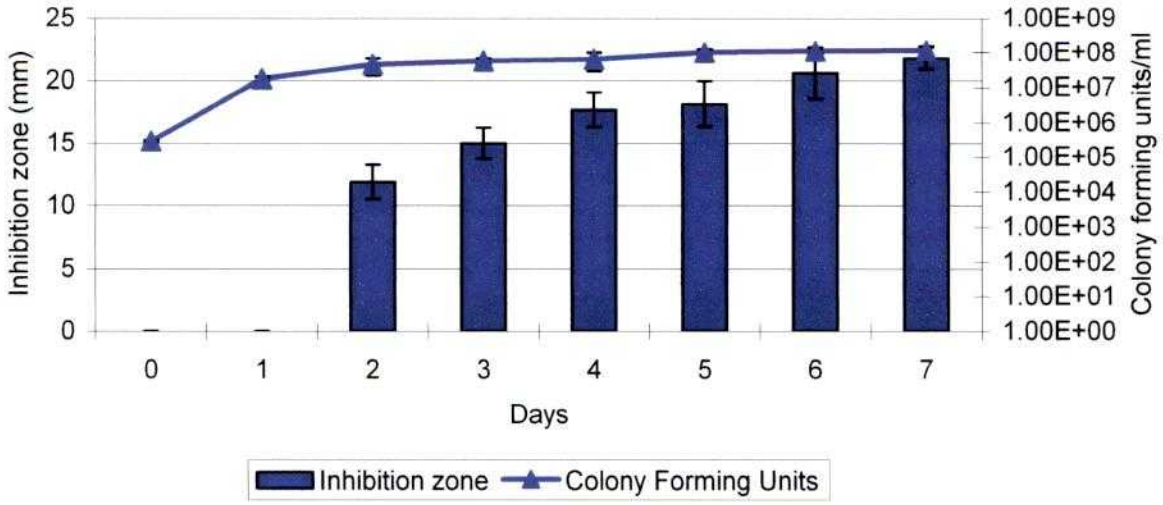

Figure 6.1: Inhibition zones formed at specific cell concentrations during continuous culturing of *Bacillus subtilis* over a seven day period.

4.3. Separation of free acid phenolic compounds by thin layer chromatography

Both water and BAW did not result in any separation of bands. The best solvent proved to be AMW. The free acid fraction was separated on pre-coated silica glass plate using AMW as solvent (Figure 6.2). Four spots were observed under UV illumination at 254 nm. No spots were noticed in the control sample. The Rf values of each of the spots are summarised in Table 6.2. As the concentration of the applied sample increased, the spot migrated further up the plate and in the 30 µl sample, spots c and d migrated together.

Table 6.2: Rf values of fluorescent spots separated using acetic acid : methanol : water as solvent on glass thin layer chromatograph plates

Observed fluorescent spot	Rf Value on silica plates using AMW	Colour at 254 nm UV illumination
a	0.22	Blue
b	0.31	Pink-red
c	0.59	Blue
d	0.64	Blue

Rf = mobility relative to front

4.4. Antifungal activity of separated fluorescent spots against Cladosporium cladosporioides

Growth of C. cladosporioides was inhibited by spots originating in the free acid fraction at the three concentrations tested. The inhibition zone corresponded to spots numbered a and b in Figure 6.3. As the applied concentration increased, the inhibitory activity also increased. Spot c and d combined also formed an inhibition zone.

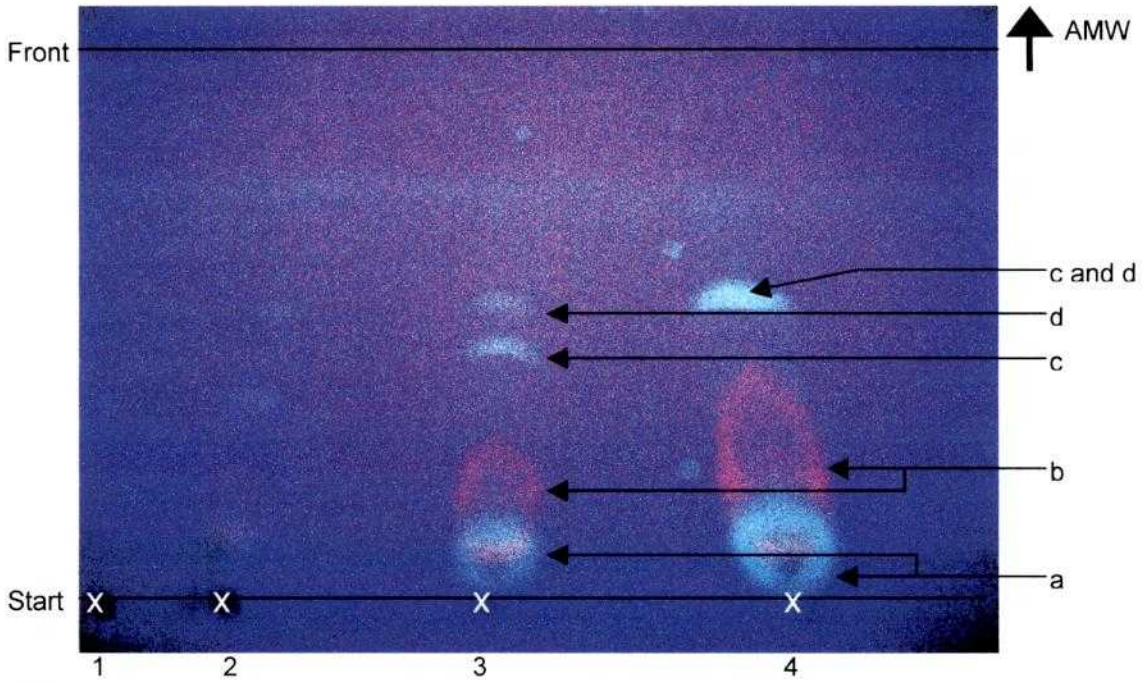

Figure 6.2: Free acid fraction of *Bacillus subtilis* growth media and control separated by thin layer chromatography using a silica glass plate, run with acetic acid:methanol:water (8:1:1, v/v/v) and viewed under UV light (254 nm) to separate potential inhibitory phenolic substances.
(No. 1 = control at 20 µl, no. 2 = 10 µl free acid extract, no. 3 = 20 µl free acid extract and no. 4 = 30 µl free acid extract)

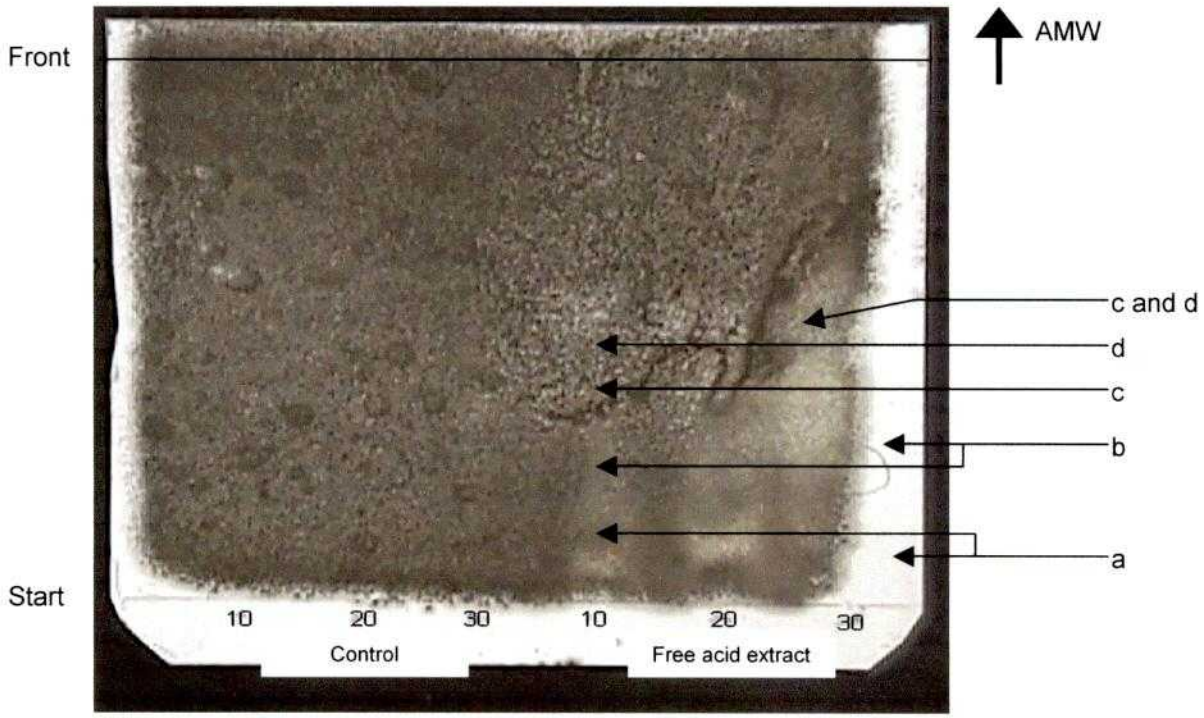

Figure 6.3: Inhibition due to spots of free acid phenolic compounds of *Bacillus subtilis* growth media separated by thin layer chromatography using a silica glass plate run with acetic acid:methanol:water (8:1:1, v/v/v) against *Cladosporium cladosporioides*.

4.5. High performance liquid chromatography of free acid phenolic compounds

In the HPLC analysis, eight peaks were observed at 325 nm while 13 peaks were evident at 280 nm (Figure 6.4). Of these 21 peaks, ten peaks coincided (1 and 2, 8 and 9, 11 and 12, 13 and 14, as well as 16 and 17). No peaks were observed at 430 nm. Table 6.3 summarises the time each of these peaks were observed. The percentage area provides an idea of how prominent a peak was and was calculated as the area covered by a peak compared to the whole area covered at a specific absorbency. The largest peaks were 2, 3, 6, 7, 11, 13 and 17.

Table 6.3: Retention time of the constituents of free acid phenolic extract determined using high performance liquid chromatography

Peak	RT (min)	Maximum Absorbency (nm)	% Area occupied	Resolved
1	2.306	280	6.00 + 3.38	No
2	2.376	325	18.10	Yes
3	3.046	280	49.77	Yes
4	3.058	325	4.44	Yes
5	4.140	325	5.15	Yes
6	5.296	280	18.59	Yes
7	5.373	325	11.18	Yes
8	7.213	280	1.45	Yes
9	7.264	325	8.65	Yes
10	8.450	280	1.51	Yes
11	8.929	325	12.18	Yes
12	8.935	280	0.95	Yes
13	9.661	325	21.68	Yes
14	9.668	280	3.41	Yes
15	12.050	280	0.60	Yes
16	14.132	280	1.01	Yes
17	14.134	325	18.61	Yes
18	15.147	280	3.58	No
19	15.573	280	3.80	No
20	15.973	280	3.51	No
21	16.547	280	2.47	No

RT = Retention time

5. DISCUSSION

A cell-free culture medium of *B. subtilis* produced a visible inhibition zone on PDA against *C. gloeosporioides* after day three of fermenting using the agar plate well method. The observed inhibition zone might have been due to the production of one or more inhibitory substances. It has previously been shown that antifungal metabolites were produced by *B. subtilis* (Chapter 4). Antibiotics reported to be produced by *B. subtilis* are most commonly peptides and lipopeptides (Peypoux *et al.*, 1984; Loeffler *et al.*, 1986; Peypoux *et al.*, 1986; Gueldner *et al*, 1988, Jacques *et al.*, 1994; Chen *et al.*, 1995; Eshita *et al.*, 1995; Kajimura *et al.*, 1995; Tsuge *et al.*, 1996; Lin *et al.*, 1998). However, Pinchuk *et al.* (2002) showed that phenolic compounds such as amicoumacin are also produced *B. subtilis*.

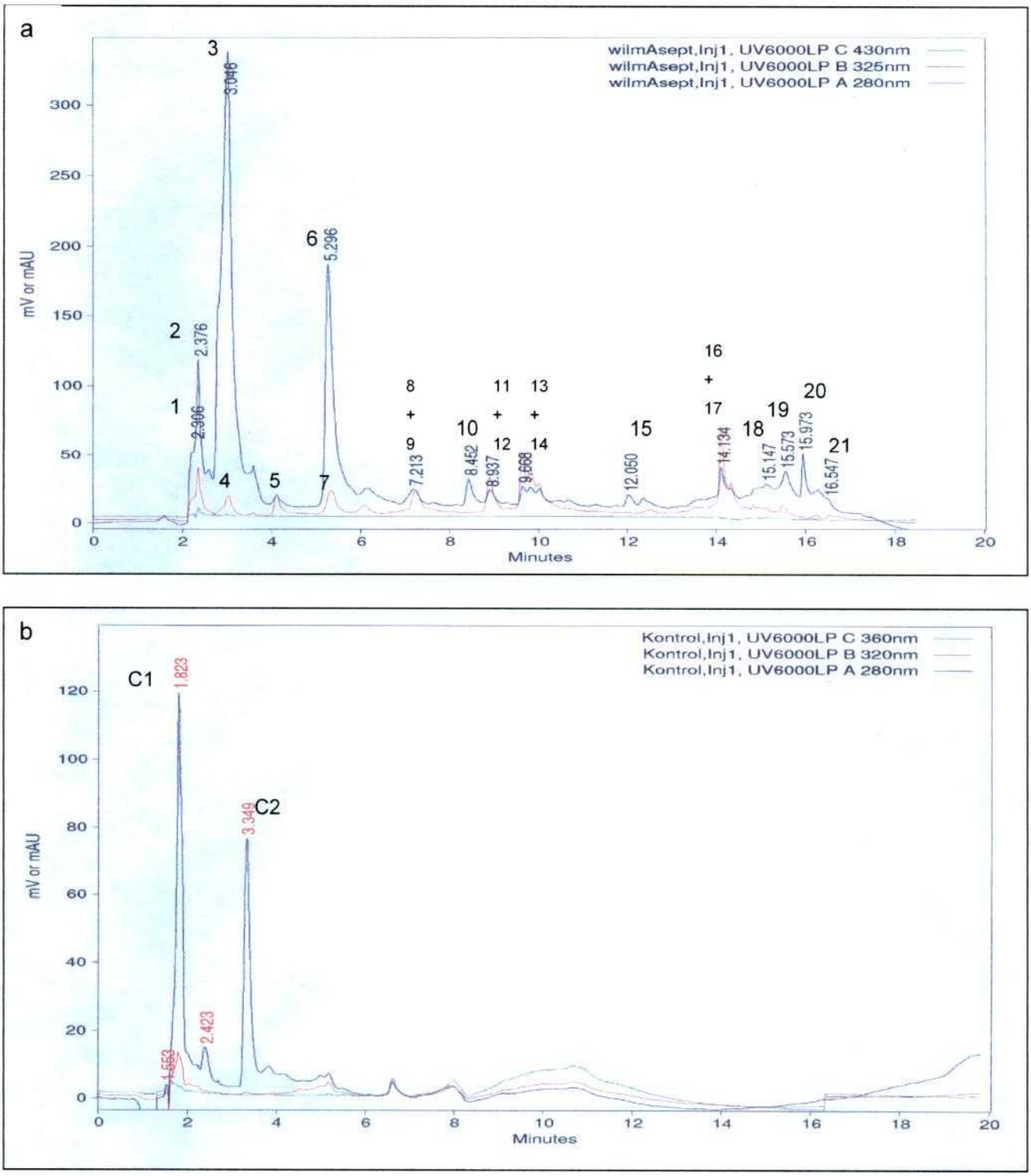

Figure 6.4: High performance liquid chromatographs of a) free acid phenolic extract viewed at 430, 325 and 280 nm, and b) control viewed at 360, 320 and 280 nm.

This study proved that *B. subtilis* secreted free acids into the growth medium. Pinchuk *et al.* (2002) also found free acids excreted by a *B. subtilis* isolate. In this study, analysis using TLC showed that four different compounds (a, b, c and d) were produced and that a and b were inhibitory to *C. cladosporioides,* a standard test organism for antifungal metabolites on TLC plates. Compounds c and d alone did not affect the fungal

growth, but the combination of c and d at the higher concentration did. Substance a was active at a concentration equivalent to 141.2 µg gallic acid ml^{-1} and b at a concentration equivalent to 211.8 µg gallic acid ml^{-1}.

Identification of the family of molecules produced by *B. subtilis* is possible if the visible spectral range and observed colours during UV illumination are kept in mind. The pink to mauve fluorescence under UV is usually associated with the coumaric group of compounds that include ferulic, sinapic, caffeic and ρ-coumaric acid (Harborne, 1984). Spot b presents this type of fluorescence and could then be one compound of the coumaric family. The HPLC analysis confirmed the production of at least eight compounds. Peak 17 (RT = 14 min), which represents a maximum absorption at 320 nm, could be ferulic acid as the coinjection with a standard of ferulic acid resulted in only one higher peak at the same time. Ferulic acid is mostly produced by plant roots and decomposing plant residues and plays a role in the protection of the plant cell wall (Caspersen *et al.*, 2000). Ferulic, ρ-coumaric, o-coumaric, caffeic, coumarin and umbelliferone are known to be germination inhibitors of wheat rust uredospores (Towers & Yamamoto, 1985). In contrast to coumaric acids, cinnamic acids are known to give two spots when chromatographed in aqueous solvents. They separate into the *cis*- and *trans*-isomers, explaining the formation of spots c and d (Harborne, 1984; Towers & Yamamoto, 1985). Moreover, another *B. subtilis* isolate was found that produced amicoumacin, a member of the isocoumarin or coumaric acid group (Pinchuk *et al.*, 2002).

Future studies should focus on the further identification of the inhibitory phenolic substances as well as their effect on fungal postharvest avocado pathogens. Since a variety of antifungal metabolites may be produced, the presence of antifungal peptide and lipopeptides should also be investigated. Even though the production of these antifungal metabolites is observed *in vitro*, their role in the *in vivo* inhibitory activity of *B. subtilis* against postharvest pathogens of avocado needs to be investigated.

6. REFERENCES

Caspersen, S., Alsanius, B.W., Sundin, P. & Jensén, P. 2000. Bacterial amelioration of ferulic acid toxicity to hydroponically grown lettuce (*Lactuca sativa* L.). Soil Biology and Biochemistry 32: 1063 – 1070.

Chen, C.L., Chang, L.K., Chang, Y.S., Liu, S.T. & Tschen, J.S.M. 1995. Transposon mutagenesis and cloning of the genes encoding the enzymes of fengycin biosynthesis in *Bacillus subtilis*. Molecular and General Genetics 248: 121 – 125.

Eshita, S.M., Roberto, N.H., Beale, J.M., Mamiya, B.M. & Workman, R.F. 1995. Bacillomycin L_c, a new antibiotic of the iturin group: Isolations, structures, and antifungal activities of the congeners. The Journal of Antibiotics 48: 1240 – 1247.

Fiddaman, P.J. & Rossall, S. 1993. The production of antifungal volatiles by *Bacillus subtilis*. Journal of Applied Bacteriology 74: 119 – 126.

Gueldner, R.C., Reilly, C.C., Pusey, P.L., Costello, C.E., Arrendale, R.F., Cox, R.H., Himmelsbach, D.S., Crumley, F.G. & Cutler, H.G. 1988. Isolation and identification of iturins as antifungal peptides in biological control of peach brown rot with *Bacillus subtilis*. Journal of Agricultural and Food Chemistry 36: 366 – 370.

Harborne, J.B. 1984. Phytochemical methods: a guide to modern techniques of plant analysis. Chapman & Hall, New York.

Havenga, W., De Jager, E.S. & Korsten, L. 1999. Factors affecting biocontrol efficacy of *Bacillus subtilis* against *Colletotrichum gloeosporioides*. South African Avocado Growers' Association Yearbook 22: 12 – 20.

Homans, A.L. & Fuchs, A. 1970. Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. Journal of Chromatography 51: 327 – 329.

Ikediugwu, F.E.O., Emoghene, A.O. & Ajiodo, P.O. 1994. Biological control of the shoot diseases *Amaranthus hybridus* caused by *Choanephora cucurbitarum* with *Bacillus subtilis*. Journal of Horticultural Science 69: 351 – 356.

Jacques, P., Hbid, C., Vanhentenryck, F., Destain, J., Baré, G., Razafindralambo, H., Paquot, M. & Thonart, P. 1994. Quantitative and qualitative study of the production of broad-spectrum antifungal lipopeptides from *Bacillus subtilis* S499. Pages 1067 – 1070 In: ECB6: Proceedings of the 6th European Congress on Biotechnology. Alberghina, L., Frontali, L. & Sensi, P. (Eds). Elsevier Science, B. V.

Kajimura, Y., Sugiyama, M. & Kaneda, M. 1995. Bacillopeptins, new cyclic Lipopeptide antibiotics from *Bacillus subtilis* FR-2. The Journal of Antibiotics 48: 1095 – 1103.

Katz, E. & Demain, A.L. 1977. The peptide antibiotics of *Bacillus*: Chemistry, biogenesis, and possible functions. Bacteriological Reviews 41: 449 – 474.

Klein, C., Kaletta, C. & Entian, K. –D. 1993. Biosynthesis of the lantibiotic subtilin is regulated by a Histidine kinase/response regulator system. Applied and Environmental Microbiology 59: 296 – 303.

Korsten, L. 1993. Biological Control of Avocado Fruit Diseases. Ph.D. thesis, University of Pretoria, Pretoria.

Korsten, L. & de Jager, E.E. 1995. Mode of action of *Bacillus subtilis* for control of avocado post-harvest pathogens. South African Avocado Growers' Association Yearbook 18: 124 – 130.

Korsten, L., de Villiers, E.E., de Jager, E.S., Cook, N. & Kotzé, J.M. 1991. Biological control of avocado postharvest diseases. South African Avocado Growers' Association Yearbook 14: 57 – 59.

Korsten, L., de Villiers, E.E., Rowell, A. & Kotzé, J.M. 1993. Postharvest biological control of avocado fruit diseases. South African Avocado Growers' Association Yearbook 16: 65 – 69.

Korsten, L, Towsen, E. & Claasens, V. 1998. Evaluation of Avogreen as post-harvest treatment for controlling anthracnose and stem-end rot on avocado fruit. South African Avocado Growers' Association Yearbook 21: 83 – 87.

Kugler, M., Loeffler, W., Rapp, C., Kern, A. & Jung, G. 1990. Rhizocticin A, an antifungal phosphono-oligopeptide of *Bacillus subtilis* ATCC 6633: Biological properties. Archives of Microbiology 153: 276 – 281.

Leifert, C., Li, H., Chidburdee, S., Hampson, S., Workman, S., Sigee, D., Epton, H.A.S. & Harbour, A. 1995. Antibiotic production and biocontrol activity by *Bacillus subtilis* CL27 and *Bacillus pumilus* CL45. Journal of Applied Bacteriology 78: 97 – 108.

Lin, G.H., Chen, C.L., Tschen, J.S.M., Tsay, S.S., Chang, Y.S. & Liu, S.T. 1998. Molecular cloning and characterization of fengycin synthetase gene *fenB* from *Bacillus subtilis*. Journal of Bacteriology 180: 1338 – 1341.

Loeffler, W., Tschen, S. -M., Vanittanakom, N., Kugler, M., Knorpp, E., Hsieh, T. –F. & Wu, T. –G. 1986. Antifungal effects of bacilysin and fengymycin from *Bacillus subtilis* F-29-3: A comparison with activities of other *Bacillus* antibiotics. Journal of Phytopathology 115: 204 – 213.

McKeen, C.D., Reilly, C.C. & Pusey, P.L. 1986. Production and partial characterization of antifungal substances antagonistic to *Monilinia fructicola* from *Bacillus subtilis*. Phytopathology 76: 136 - 139.

Peypoux, F., Pommier, M.T., Das, B.C., Besson, F., Delcambe, L. & Michel, G. 1984. Structures of bacillomycin D and bacillomycin L peptidolipid antibiotics from *Bacillus subtilis*. The Journal of Antibiotics XXXVII: 1600 – 1604.

Peypoux, F., Pommier, M.T., Marjon, D., Ptak, M., Das, B.C. & Michel, G. 1986. Revised structure of Mycosubtilin, a peptidolipid antibiotic from *Bacillus subtilis*. The Journal of Antibiotics XXXIX: 636 – 641.

Pinchuk, I.V., Bressollier, P., Sorokulova, I.B., Verneuil, B. & Urdaci, M.C. 2002. Amicoumacin antibiotic production and genetic diversity of *Bacillus subtilis* strains isolated from different habitats. Research in Microbiology 153: 269 – 276.

Podile, A.R. & Prakash, A.P. 1996. Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF1. Canadian Journal of Microbiology 42: 533 – 538.

Pusey, P.L., Wilson, C.L., Hotchkiss, M.W. & Franklin, J.D. 1986. Compatibility of *Bacillus subtilis* for postharvest control of peach brown rot with commercial fruit waxes, Dichloran, and cold-storage conditions. Plant Disease 70: 587 – 590.

Sailaja, P.R., Podile, A.R. & Reddanna, P. 1997. Biocontrol strain of *Bacillus subtilis* AF1 rapidly induces lipoxygenase in groundnut (*Arachis hypogaea* L.) compared to crown rot pathogen *Aspergillus niger*. European Journal of Plant Pathology 104: 125 – 132.

Sakajoh, M., Solomon, N.A. & Demain, A.L. 1987. Cell-free synthesis of the dipeptide antibiotic bacilysin. Journal of Industrial Microbiology 2: 201 – 208.

Shoji, J. 1978. Recent chemical studies on peptide antibiotics from the genus *Bacillus*. Advances in Applied Microbiology 24: 187 – 214.

Swain, T & Hillis, W.E. 1959. The phenolic constituent of *Prunus domestica* I. The quantitative analysis of phenolic constituents. Journal of Science of Food and Agriculture 10: 63 – 68.

Towers, G.H.N & Yamamoto, E. 1985. Interactions of cinnamic acid and its derivatives with light. Pages 271 – 287 In: Annual proceedings of the phytochemical society of Europe: the biochemistry of plant phenolics. Van Sumere, C.F. & Lea, P.J. (Eds). Clarendon Press, Oxford.

Tsuge, K., Ano, T. & Shoda, M. 1996. Isolation of a gene essential for biosynthesis of the lipopeptide antibiotics plipastatin B1 and surfactin in *Bacillus subtilis* YB8. Archives of Microbiology 165: 243 – 251.