Chapter 3

Characterisation of low molecular weight compounds isolated from *Pyrenophora teres*

3.1 Introduction

Previous research isolated toxic compounds from *Pyrenophora teres* which belong to several chemical classes; marasmines (Gaumann and Jaag 1946) such as toxins A, B and C (Smedegaard-Petersen 1977b, Bach *et al*. 1979, Friis *et al*. 1991); pyrenolines (Coval *et al*.1990) and pyrenolides (Nukina *et al*. 1980, Nukina *et al*. 1980, Nukina and Hirota 1992).

Toxin A is *N*-(2-amino-2-carboxyethyl) aspartic acid and has been isolated from *P. teres* but not other natural sources (Figure 3.1). Four stereoisomers of this toxin have been synthesised chemically using maleic acid and D and L-diaminopropionic acids (Friis *et al.* 1991). Toxin C is *N*-[2-(2-amino-2-carboxyethyl-amino)-2-carboxyethyl] aspartic acid (Figure 3.1) and is identical with aspergillomarasmine A isolated from cultures of *Aspergillus flavus oryzae* (Haenni *et al*. 1965, Robert *et al*. 1962) *Colletotrichum gloeosporioides* (Bousquet *et al*. 1971) and *Fusarium oxysporum* f. sp. *melonis* (Trouvelot *et al*. 1971, Camporota *et al*. 1973). Toxin B is 1-(2-amino-2-carboxyethyl)-6-carboxy-3-carboxymethyl-2-piperazinone and identical to anhydroaspergillomarasmine A, a lactam of aspergillomarasmine A (Figure 3.1). Toxins A and B have also been isolated from barley leaves inoculated with *P. teres* (Smedegaard-Petersen 1977b). These three toxins (A, B and C) showed phytotoxicity on a range of different plant species (Smedegaard-Petersen 1977b, Bach *et al.* 1979).

Additionally, two isoquinoline fungal derivatives termed pyrenoline-A and B have also been isolated from culture filtrates of *P. teres* (Figure 3.2). Pyrenoline A and pyrenoline B both showed phytotoxicity on different plant species including different varieties of barley (Coval *et al*. 1990).

Further, some cytotoxic metabolites termed pyrenolides A (Nukina *et al.* 1980), B, C (Nukina *et al.* 1980) and D (Nukina and Hirota 1992) have also been isolated hyphae in several fungal species (Nukina *et al.* 1980) while pyrenolide B and pyrenolide C also showed cytotoxicity on fungal species including *Cochliobolous lunata* (Nukina *et al*. 1980). However, the tricyclic pyrenolide D does not appear to affect fungi, but is cytotoxic to human promyelocytic leukaemia cell line (HL-60 cells) (Nukina and Hirota 1992).

In this study, the partially purified LMWCs isolated from culture filtrates of *Pyrenophora teres* f. *teres* (*Ptt*) and *Pyrenophora teres f. maculata* (*Ptm*) (chapter 2) were further characterised. Additionally, the LMWCs were purified, identified and the biological activity of each individual compound was also examined on a susceptible cultivar of barley (Sloop). Furthermore, Toxin A which is the direct precursor of other marasmines (Friis *et al.* 1991), was chemically synthesised and then used as a substrate to synthesise a compound isolated in this study which shows no resemblance to previously described compounds from *P. teres*.

Figure 3.1 The biosynthetic pathway for *P. teres* toxins and related phytotoxins. Conversions marked with arrows have been shown to occur in the cultures of *P. teres* (Friis *et al.* 1991).

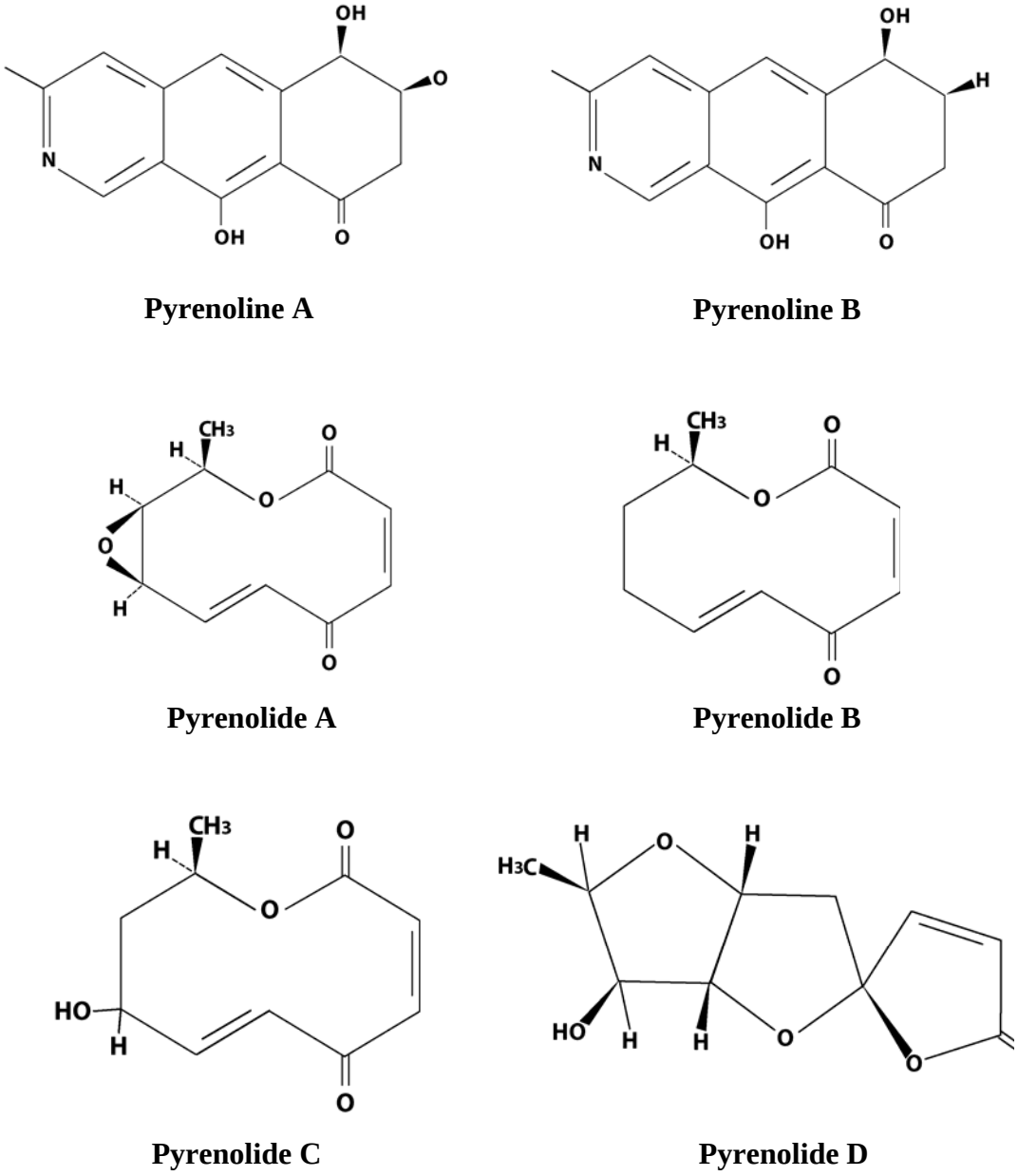


Figure 3.2 Chemical structures of cyclic phytotoxins (pyrenoline A and B; Coval *et al.* 1990) and toxins (Pyrenolides A; Nukina *et al.* 1980, B, C; (Nukina *et al.* 1980, and D; Nukina and Hirota 1992) isolated from *P. teres*.

3.2 Materials and methods

3.2.1 Initial characterisation of partially purified LMWCs extracted from *Ptt* and *Ptm* culture filtrates

Partially purified LMWCs extracted from culture filtrates of *Ptt* and *Ptm* grown in PFCM (section 2.2.7) were loaded (2 µL) onto a sheet of Whatman No. 1 filter paper (15.5 × 28.5 cm) and run in formic acid and acetic acid buffer (pH= 1.75 as described in section 2.2.8) in a solvent-cooled HVPE device at 3000 volts for 90 minutes (Tate 1968). The paper was then air-dried, stained with 0.2 % ninhydrin in acetone and heated at 100 °C for 2 to 3 min to visualise ninhydrin-positive compounds (Weiergang *et al.* 2002b). The experiment was conducted three times in the same running conditions. The mobility of each ninhydrin-positive compound was determined relative to aspartic acid for both forms of the fungus. LMWCs extracted from either *Ptm* or *Ptt* showed similar patterns on HVPE. LMWCs extracted from either *Ptt* or *Ptm* showed similar relative mobility. However, LMWCs extracted from *Ptm* showed stronger reaction with ninhydrin and therefore these metabolites were further characterised.

3.2.2 Host range of partially purified LMWCs

Seeds (5-6) of wheat (*Triticum aestivum* L.), faba bean (*Vicia faba* L.), tobacco (*Nicotiana tabacum* L.) as well as a susceptible barley cultivar (Sloop) and a resistant line (CI9214) were sown in 80×100 mm pots as described in section 2.2.1. To determine the host specificity, partially purified LMWCs (150 µL) extracted from *Ptm* were injected to the second and third leaves of abovementioned plants at 3-4 leaf stage using the Hagborg device as described earlier (section 2.2.6). Two leaves of two individual plants were treated in two independent experiments. Similar to the

pathogenicity test and previous bioassays (Chapter 2), plants were kept under cover overnight in a growth chamber at 18 to 22 ^{o}C and then maintained in a 16 h light and 8 h dark photoperiod and monitored daily. Samples were collected after 120 h and photographed by a scanner (Epson reflection 4180 photo scanner) as per section 2.2.12.

3.2.3 Effect of light during incubation on biological activity of LMWCs

Partially purified LMWCs (150 µL) were injected into the second leaves of eight individual barley plants (Sloop) at Zadoks' growth stage 14 as described previously (chapter 2, section 2.2.6). Four plants were kept in the dark constantly and the rest as per section 3.2.2 in a growth chamber at 18 to 22 ^{o}C in a 16 h light and 8 h dark photoperiod. Control plants were injected with sterile distilled water (SDW) and kept in the constant dark or the photoperiod described above. Treated leaves were collected after 144 h and photographed by a scanner (Epson reflection 4180 photo scanner). The experiment was conducted twice so that a total of eight leaves were treated with each sample.

3.2.4 Effect of heat and acid hydrolysis on biological activity of LMWCs

Partially purified LMWCs were heat-treated at 100 ^{o}C for 1 h and autoclaved at 121 ^{o}C for 30 min. They were also hydrolysed using 5 M hydrochloric acid at 130 ^{o}C for 6 h. The acid was then removed by drying down the samples using a speed vacuum concentrator device (Speedivac, Savant Instruments Inc. Hicksnile, New York, USA). The pH of all samples was adjusted to 7 using a few drops of 0.1 % ammonia. The samples were then injected into second leaves of barley at Zadoks' growth stage 14 as described earlier (section 2.2.6). Two leaves of two individual plants were treated in two independent experiments. The plants were then kept under

the conditions described above (section 3.2.2) and monitored every 24 h. Samples were collected after 120 h and photographed using a scanner (Epson reflection 4180 photo scanner) as per section 2.2.12.

3.2.5 Effect of temperature during incubation on biological activity of partially purified LMWCs

Partially purified LMWCs (150 µL) were injected into the first and second leaves of two individual barley plants (cv. Sloop) at Zadoks' growth stage 14 as described previously (section 2.2.6). Two plants were kept in a cold room at constant 4 ^{o}C and two plants were kept in a growth chamber at 18 to 22 ^{o}C. Control plants were injected with sterile distilled water and kept in the cold (4 ^{o}C) or at 18 to 22 ^{o}C. All treated samples were kept under 16 h light and 8 h dark photoperiod described previously (section 2.2.6). Treated plants were kept in cold conditions for 168 h to ensure enough time was allowed for symptom expression and then photographed by a scanner (Epson reflection 4180 photo scanner) as per section 2.2.12.

3.2.6 Purification of LMWCs

Although the ninhydrin positive spots in the electrophoretogram (as per section 3.2.1) were the same in extracts from both fungi, they were stronger in *Ptm*. Hence, the LMWCs were further characterised and purified from *Ptm* using available technique such as HVPE. LMWCs (100 µL) were loaded on a sheet of Whatman No. 1 filter paper (15.5 × 28.5 cm) as a series of individual spots in which each spot contained 3 µL of LMWCs. The paper was run under the same conditions described in section 3.2.1 for 100 minutes (Tate 1968). The paper was then blotted between Whatman No. 3 filter papers to absorb excess buffer and air-dried. A one centimetre strip on both edges of the paper was cut, observed against an ultraviolet (UV) source

(UVGL-25 multi-band 254/365 nm, 215-250 volts, 50/60Hz, 0.12 Amps, Mineralight® Lamp, San Gabriel, California, USA), and then stained in 0.2 % ninhydrin in acetone. The paper was then cut into strips based on the position of each individual ninhydrin positive spot or UV absorbent metabolites on the edges of the paper. The metabolites were extracted from the paper strips using 0.1 % ammonia solution and centrifugation at 13000 rpm for 5 minutes. The extracts were then lyophilised until dryness and redissolved in 500 µL distilled water containing 0.1 % ammonia. The whole partially purified LMWCs extracted from *Ptm* grown on PFCM (section 2.2.7) were loaded onto several papers and run under the same conditions to collect enough purified metabolites for bioassay and further characterisation. To check the purity and relative mobility, 1 µL of individual LMWCs was run on a sheet of Whatman No.1 paper in the same running conditions described above (Tate 1968).

3.2.7 Bioassay of individually purified LMWCs

Excised barley leaves were treated with each individually purified LMWC at Zadoks' growth stage 14 (as per section 2.2.6). The second leaf of barley plants was cut under water and placed in 1.5 mL of sterile distilled water containing 150 µL of each purified LMWC in a test tube. The excised leaves were first allowed to take up the solution and subsequently SDW was added when necessary. The samples were monitored to make sure there was enough water in the test tubes during the whole period of the experiment. SDW and the extract of a blank paper run under the same conditions as LMWCs (section 3.2.6) were used to treat the leaves as controls. The samples were then placed in a growth chamber under a 16 h light and 8 h dark photoperiod at 18 to 22 °C. Treated leaves were then examined every 24 h for 144 h and classified as (+) if symptoms were present, (-/+) if weak symptoms were observed

and (-) if no symptoms were evident. Two leaves were treated with each purified LMWC in each of three separate experiments (n=6).

3.2.8 Putative identification of purified LMWCs

Individual purified LMWCs were identified using mass spectrophotometry, electrophoretogram properties and comparison of the data with previously described LMWCs from *P. teres* (Bach *et al*. 1979, Friis *et al*. 1991, Smedegaard-Petersen 1977b, Weiergang *et al*. 2002b, Nukina *et al*.1980, Nukina *et al*. 1980, Nukina and Hirota 1992, Coval *et al*. 1990).

Mass spectrometric analysis was carried out with an API-300 mass spectrometer equipped with an ionspray ion source (PE Sciex, Thornhill, Ontario, Canada) at the Australian Wine Research Institute. In positive ion mode, the spray needle, orifice and ring were set at potentials of 5 kV, 35 V and 250 V respectively. The curtain (nitrogen) and nebuliser (air) gases were set at 9 and 12 units, respectively. Sample solution was loaded using a flow injector (Rheodyne model 8125, USA) fitted with a 5 µL loop. Solvent (0.5% formic acid: 49.5% distilled water: 50% acetonitrile) was used to deliver the sample solution into the mass spectrometer at a flow rate of 5 µL/min by a 140B Solvent Delivery System (Applied Biosystems, USA). Positive ion mass spectra were recorded in a range of mass-to-charge ratio (*m/z*) from 100 to 1000. Mass spectrometric data were processed using Analyst 1.4 (PE Sciex). In negative ion mode, the spray needle, orifice and ring were set at potentials of -4.5 kV, -35 V and -250 V, respectively. Solvent (0.1% ammonia: 49.9% distilled water: 50% acetonitrile) was used to deliver the sample solution into the mass spectrometer. The other conditions were the same as those for the positive ion mode. Mass signals below 150

m/z were not considered due to possible contaminations (Tate, personal communication).

Mobility of each ninhydrin positive spot was determined relative to aspartic acid after running the purified LMWCs (4 µL) in HVPE in the same running conditions described previously (section 3.2.1).

One of the LMWCs isolated in this study (S-7) was further characterised. The absorbance of three different concentrations (25, 50 and 100 mM) of this compound was measured between 220 and 280 nm using a spectrophotometer (Metertech UV/VIS SP8001) as per section 2.2.5 and compared with those of phenylalanine (6.25, 12.5, 25 and 100 mM). Additionally, the compound was acid hydrolysed using 5 M hydrochloric acid at 130 °C for 6 h, run on HVPE and stained with ninhydrin as per section 3.2.1.

3.2.9 Chemical synthesis of Toxin A and a novel derivative

Because only trace amounts of Toxin A were isolated from *P. teres* in this study, the toxin was synthesised chemically using a method modified from a previously established protocol (Friis *et al.* 1991). This was then used to synthesise the novel compound detected in this study.

3.2.9.1 Preliminary optimisation of conditions for Toxin A synthesis

Because HVPE samples produced via the Friis et al (1991) protocol indicated a relatively poor yield of the putative Toxin A, the toxin synthesis was optimised. Initially, maleic acid di-sodium salt (0.3 mM, Sigma-Aldrich, St Louis, Montana, USA) and DL- 2,3 diaminopropionic acid monohydrochloride (DAP, 0.1mM, Sigma-

Aldrich) were dissolved in distilled water (1 mL) at pH 6.2 and incubated at 60 and 80 °C for up to 20 h.

Since a large quantity of DAP was left unreacted in the reaction mixture, the amounts of maleic acid in the reaction were increased to 1 mM. Additionally, to eliminate the effect of pH changes, the reaction was conducted under buffered conditions. Two buffers (pH 8.04 and pH 7.73) were made using 66 mM of potassium di-hydrogen orthophosphate (Merck, Victoria, Australia) and 66 mM di-sodium hydrogen orthophosphate (Merck) (Vogel 1944). The reaction mixture (3.2 mL) was incubated at 60, 80, 100 and 120 °C for up to 15 h.

In a further synthesis experiment, the conditions for reaction were also optimised for Toxin A synthesis using both D and L-2,3diaminopropionic acid monohydrochloride (Sigma-Aldrich, D and L-DAP). Maleic acid (0.5 mM) was mixed with D and L-DAP (0.05 mM) in the 160 µL of buffer (pH 8.04) and incubated at 60 and 70 °C for up to 48 h. Since some degradation was still observed in the reaction mixture at 60 °C after 24 h, Toxin A was considered to be optimally synthesised using L or D-DAP at 57 °C for 27 h.

In all synthesis experiments, aliquots (10 µL) were collected at different time points and monitored for ninhydrin-positive compounds by HVPE (pH 1.75) at 400 V for 20 min (as per section 2.2.8).

3.2.9.2 Larger scale synthesis of D and L isomers of Toxin A

Once the conditions of the reaction had been optimised, Toxin A was prepared on a larger scale using either L-DAP(1 mM) or D-DAP (1 mM) and maleic acid (10 mM) in 3.2 mL of the buffer (pH 8.04) mentioned in section 3.2.9.1. Both reaction mixtures

were incubated at 57 °C for 27 h and then purified as below. To remove traces of un-reacted DAP, the reaction mixture was applied to a D-50 (NH_4^+) column (Dow Chemical Company) (10 mL) in ammonium ion form. The column was washed with distilled water to remove the non-retained compounds and eluted with a gradient of ammonium acetate (10 to 100 mM). All fractions (washings and eluates) were freeze-dried before being redissolved in 100 μL distilled water containing 0.1 % ammonia. Ninhydrin-positive compounds were monitored by HVPE (pH 1.75). Fractions containing ninhydrin-positive compounds (and therefore potentially containing Toxin A) were pooled. These fractions also contained UV-absorbing materials at zero mobility which indicated the presence of maleic acid. To remove the excess maleic acid, the sample (500 μL) was subjected to a D-50 column (Dow Chemical Company) in hydrogen form [H^+], washed with distilled water and then eluted with 2 M ammonia (Smedegaard-Petersen 1977b). The eluates from the D-50 column were lyophilised and then redissolved in 100 μL of distilled water containing 0.1 % ammonia. Drop wise addition of methanol to the sample caused precipitation of a solid. The supernatant and the precipitate were monitored for Toxin A by HVPE (pH 1.75) as per section 2.2.8. A ninhydrin-positive compound with the same relative mobility as natural Toxin A was detected in the precipitate. The electrophoretically homogenous Toxin A was washed three times with methanol and dried under vacuum.

3.2.9.3 Identification of synthesised Toxin A

The identity of the synthesised isomers of Toxin A was confirmed using mass spectrometry in a positive ion mode. Samples were dissolved in a solvent (0.5% formic acid: 49.5% distilled water: 50% acetonitrile) to deliver to a API-300 mass spectrometer equipped with an ionspray ion source (PE Sciex, Thornhill, Ontario,

Canada) as described in section 3.2.8. Additionally, Ms/Ms analysis was performed for both isomers on a ThermoFinnigan LCQ Deca XP^{Plus} LCMS Ultra-sensitive ion trap equipped with an electrospray ion (ESI) source (ThermoFinnigan, San Jose, California, USA). In positive ion mode, the spray, capillary, tube lens offset, multipole 1 offset, multipole 2 offset, multipole RF amplitude, lens and lens entrance potentials were set at 4.6 kV, 26 V, 25 V, -6.5 V, -10.5 V, 400 V, -18.0 V and -50.0 V respectively. The sheath and auxiliary gases were set at 15 and 0 arbitrary units, respectively. Sample solution (dissolved in 0.5 % formic acid: 49.5 % distilled water: 50 % acetonitrile) was delivered into the mass spectrometer at a flow rate of 40 µL/min using a syringe pump (ThermoFinnigan) and a 500 µL SGE syringe (Adelab, Adelaide, SA, Australia). Positive ion mass spectra were recorded in a range of mass-to-charge ratio (*m/z*) from 90 to 400. Mass spectrometric data were processed using Xcalibur Home page version 1.3 (ThermoFinnigan).

3.2.9.4 Biological activity of synthesised isomers of toxins A

A range of concentrations (3.1 to 100 µM) of the different isomers of Toxin A (the product from DL, D and L DAP) were prepared in 1 mL distilled water containing 0.1 % ammonia and used to treat second and third excised barley leaves at Zadoks' growth stage 14 as per section 2.2.6. The experiment was conducted twice and in each individual experiment three leaves were treated with each of the isomers in each concentration. Control leaves were treated with SDW and SDW containing 0.1 % ammonia. The leaves were kept at 22 °C for 120 h (as per section 2.2.6) and images were captured using a scanner (Epson reflection 4180 photo scanner).

3.2.9.5 Chemical synthesis of a new compound from *P. teres*

Since mass spectrometry and electrophoretic properties of one of the isolated compounds (S-7) from *P. teres* showed no resemblance with previously described compounds, preliminary experiments were conducted to chemically synthesise this compound. Mass spectrum, the UV absorbance and acid hydrolysis of S-7 suggested that this compound contained an imino *N*-phenylalanine linkage to Toxin A. Consequently, to synthesise this compound, sodium phenylpyruvate (1 mM, Sigma-Aldrich) was dissolved in buffer pH 8.04 (section 3.2.8.1) and added to the L-isomer of Toxin A (0.1 mM). Sodium cyanoborohydride (Sigma-Aldrich) was then added to the reaction mixture and the reaction incubated at 22 °C for 24 h while stirring. Aliquots of the reaction (10 µL) were withdrawn after 24 h and monitored for ninhydrin positive and UV-absorbing compounds using HVPE at pH 1.75 (as per section 2.2.8).

3.3 Results

3.3.1 LMWCs extracted from *Ptt* and *Ptm* culture filtrates

Both *Ptt* and *Ptm* grown on PFCM produced eight ninhydrin-positive metabolites separated using HVPE with the same mobilities at 0.48, 0.57, 0.65, 0.67, 0.72 0.82, 1.03 and 1.14 relative to aspartic acid (R_m) (Figure 3.3). *Ptm* showed stronger spots in reaction with ninhydrin at the volumes run compared with *Ptt* and were used for further analysis.

3.3.2 Host range of partially purified LMWCs

Partially purified LMWCs showed no host specificity when injected into different plant species (Figure 3.4). All treated plants showed water soaking 24 to 48 h post treatment followed by chlorosis. Faba bean was more sensitive to the toxins since general necrosis was observed in the injection site at 24 h and in the whole of the infiltrated zone at 48 h post treatment compared with other plants which showed extreme chlorosis leading to cell death 72 to 120 h post treatment (Figure 3.4). Barley plants (Sloop and CI9214) and wheat showed water soaking 48 h post treatment in the tips of the leaves followed by general chlorosis 72 to 120 h post treatment (Figure 3.4). The susceptible cultivar (Sloop) and resistant line of barley (CI9214) showed similar sensitivity to the LMWCs with symptom expression (water soaking and chlorosis) appearing similar for both. Wheat showed less sensitivity compared with barley where chlorosis was observed in the injection site and in the tips of the treated leaves. Tobacco leaves only showed the symptoms in the injection site (Figure 3.4).

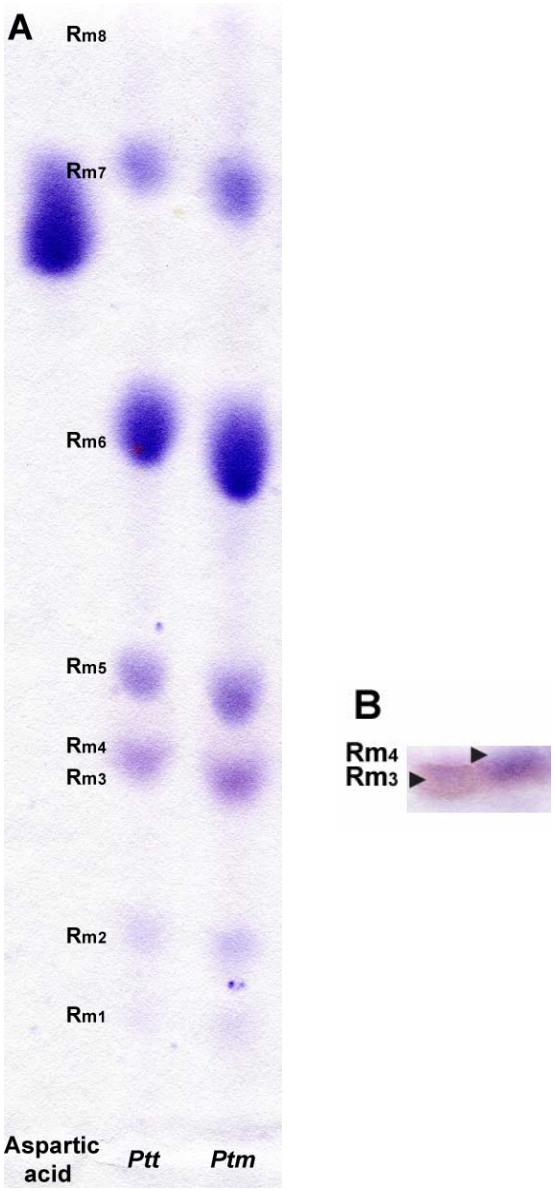


Figure 3.3 Low molecular weight compounds (LMWCs) produced by *Pyrenophora teres* f. *maculata* (*Ptm*) and *Pyrenophora teres* f. *teres* (*Ptt*). Partially purified LMWCs were subjected to high voltage paper electrophoresis and visualised using ninhydrin as described in section 3.2.1 (A). Two of the compounds were difficult to distinguish (R_{m3} and R_{m4}), when magnified (B) the reddish UV absorbing compound at R_{m3} (left) can be distinguished from R_{m4}. The electrophoretogram is representative of three individual experiments.

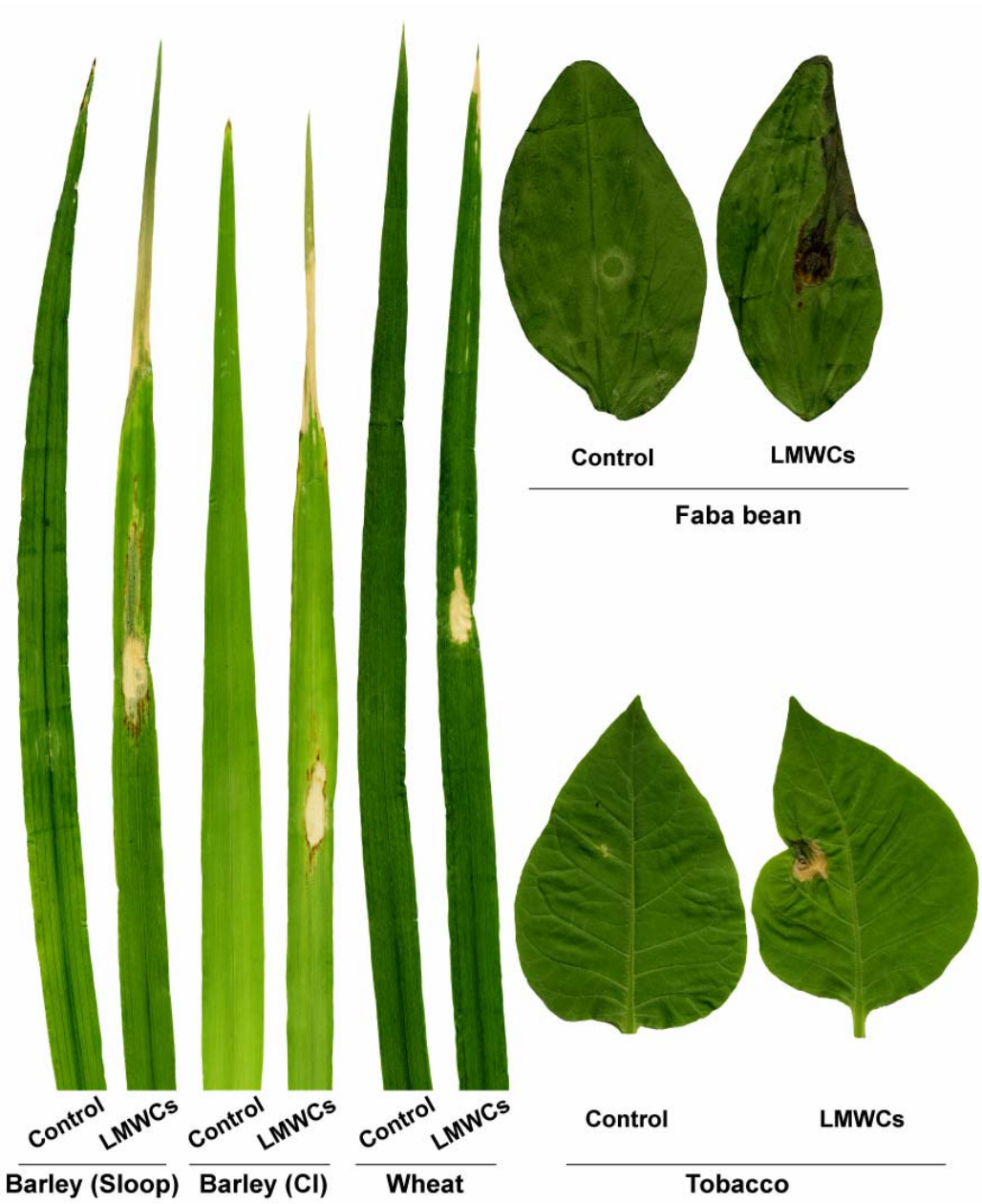


Figure 3.4 Biological activity of low molecular weight compounds (LMWCs) on different plant species. Partially purified LMWCs extracted from culture filtrates of *Pyrenophora teres* f. *maculata* (*Ptm*) and sterile distilled water (as control) were injected (150 µL) into the leaves of plant species including a susceptible cultivar (Sloop) and a resistant line of barley (CI9214) using the Hagborg device as per section 3.2.2. The leaves were photographed 120 h post treatment. Two leaves of two individual plants were treated in two independent experiments.

3.3.3 Effect of light during incubation on the biological activity of LMWCs

Partially purified LMWCs showed bioactivity only in the presence of light (usual photoperiod as per section 3.2.3) during incubation where the symptom of chlorosis was observed 72 h post treatment and progressed to its extreme level 144 h after treatment when the samples were collected for photography (Figure 3.5). Treated plants kept under constant dark conditions did not show clear symptoms but only very faint chlorosis at the injection site, which also was visible in the controls treated with SDW.

3.3.4 Effect of heat and acid hydrolysis on the biological activity of LMWCs

Autoclaved and hydrolysed LMWCs induced no symptoms while the LMWCs treated with heat at 100 ^{o}C for 1 h, still showed some phytotoxicity when injected into attached leaves where water-soaking was observed 24 h post treatment followed by chlorosis and general necrosis 72 to 120 h post treatment (Figure 3.6).

3.3.5 Effect of temperature during incubation on the biological activity of LMWCs

Partially purified LMWCs induced water soaking first in the tips of the leaves followed by general chlorosis in and above the injection site of the attached leaves kept at 22 ^{o}C. However, when plants were incubated in cold conditions (4 ^{o}C) after treatment, only water soaking was observed in and above the injection site of the treated leaves. General chlorosis did not develop even when leaves were kept for 168 h under constant cold conditions (4 ^{o}C) (Figure 3.7).

Figure 3.5 Effect of light during incubation on biological activity of partially purified low molecular weight compound (LMWCs) from *Pyrenophora teres*. Attached barley leaves were treated with LMWCs and kept under 16 h light and 8 h dark photoperiod or in the constant dark for up to 144 h as per section 3.2.3. The picture is representative of eight leaves treated in two individual experiments. Control leaves were treated with sterile distilled water (SDW).

Figure 3.6 Effect of hydrolysis and heat on biological activity of low molecular weight compounds (LMWCs) extracted from culture filtrates of *Pyrenophora teres*. Partially purified LMWCs were heated, autoclaved or hydrolysed prior to injection into attached barley leaves using the Hagborg device as described in section 3.2.4. Untreated LMWCs were used as a control. The leaves were photographed 120 h post treatment and are representative of eight leaves treated in two independent experiments.

Figure 3.7 Effect of temperature during incubation on biological activity of low molecular weight compounds (LMWCs) from *Pyrenophora teres*. Partially purified LMWCs were injected into attached barley leaves and the plants were kept at 4 or 22 ^{o}C as per section 3.2.5. To make sure no chlorosis would be induced, the leaves were collected 168h post treatment and are representative of eight leaves treated in two separate experiments (n=8).

3.3.6 Purification of LMWCs

Fourteen ninhydrin positive compounds (designated as S-1 to S-14) were observed during the purification of LMWCs in which eight compounds (S-1, S-2, S-7, S-8, S-9, S-12, S-13, S-14; Figure 3.8) were consistent in relative mobility with those observed in the starting material ($R_{m1,}$ $R_{m2,}$ R_{m3}, R_{m4}, R_{m5} R_{m6}, R_{m7}, and R_{m8} in figure 3.3). Six compounds (S-3, S-4, S-5, S-6, S-10 and S-11) were presumed to be artefacts compounds with the same mobilities were not detected in the starting materials (Figure 3.3) but were observed at the end of the purification process (Figure 3.8). For example, the first two ninhydrin positive metabolites in the starting materials (R_{m1} and R_{m2}) with mobilities of 0.48 and 0.57 (Figure 3.3) were very close to each other and thus extracted from the paper as a mixture. These two compounds were then re-run on a new sheet of the paper and re-extracted from the paper as individual purified compounds. However, six ninhydrin positive compounds resulted from these two compounds during the purification process of which four are presumed to be artefacts. Similar degradation/conversion occurred for the three compounds at R_{m3}, R_{m4}, and R_{m5} when they were re-run and re-extracted during the purification process (Figures 3.3 and 3.8). All 14 LMWCs resulted from the first and second run of the papers (S-1 to S14) were ninhydrin-positive species and turned purple except S-7 which turned reddish in reaction with ninhydrin (Figure 3.8).

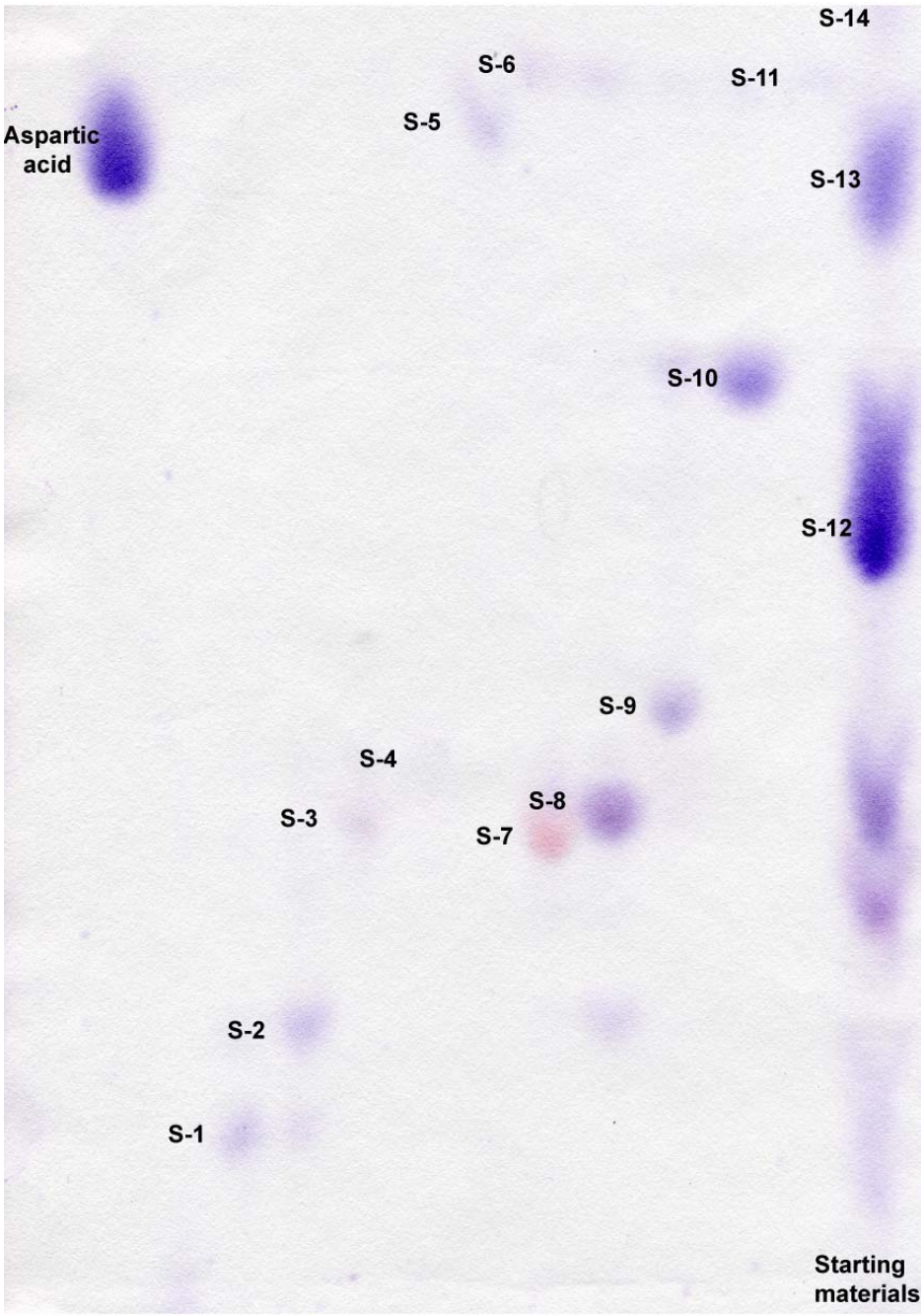


Figure 3.8 Purified LMWCs extracted from culture filtrates of *Pyrenophora teres* f. *maculata* (*Ptm*) grown on PFCM. Partially purified LMWCs were subjected to HVPE and then individual ninhydrin-positive spots from that starting material extracted as per section 3.2.6. Purified metabolites (4 µL) were subjected to HVPE and the mobility of each compound (S-1 to S-14) determined relative to aspartic acid. The paper is representative of three individual experiments.

3.3.7 Biological activity of purified LMWCs

Strong biological activity was induced by S-1, S-3, S-7, S-8, S-9, S-10 and S-12 compounds which induced water soaking and extensive chlorosis in excised leaves between 48 and 96 h post treatment. These compounds induced water soaking during the first 48 h post treatment in the tips of the leaves followed by chlorosis. The chlorosis appeared after 72 h and started at the tips of the leaves, gradually extending to their bases. S-1 did not show chlorosis but strong water soaking was observed 24 h after treatment. Excised leaves treated with S-1, then became dry while they were still green 72 h post treatment (Figure 3.9). S-2, S-13 and S-14 caused chlorosis only at the tips of the excised leaves.

Other compounds (S-4, S-5, S-6 and S-11) which were produced during the purification process induced no symptoms or very weak chlorosis at the tips of the leaves and were considered to be weakly biologically active compounds (Figure 3.9).

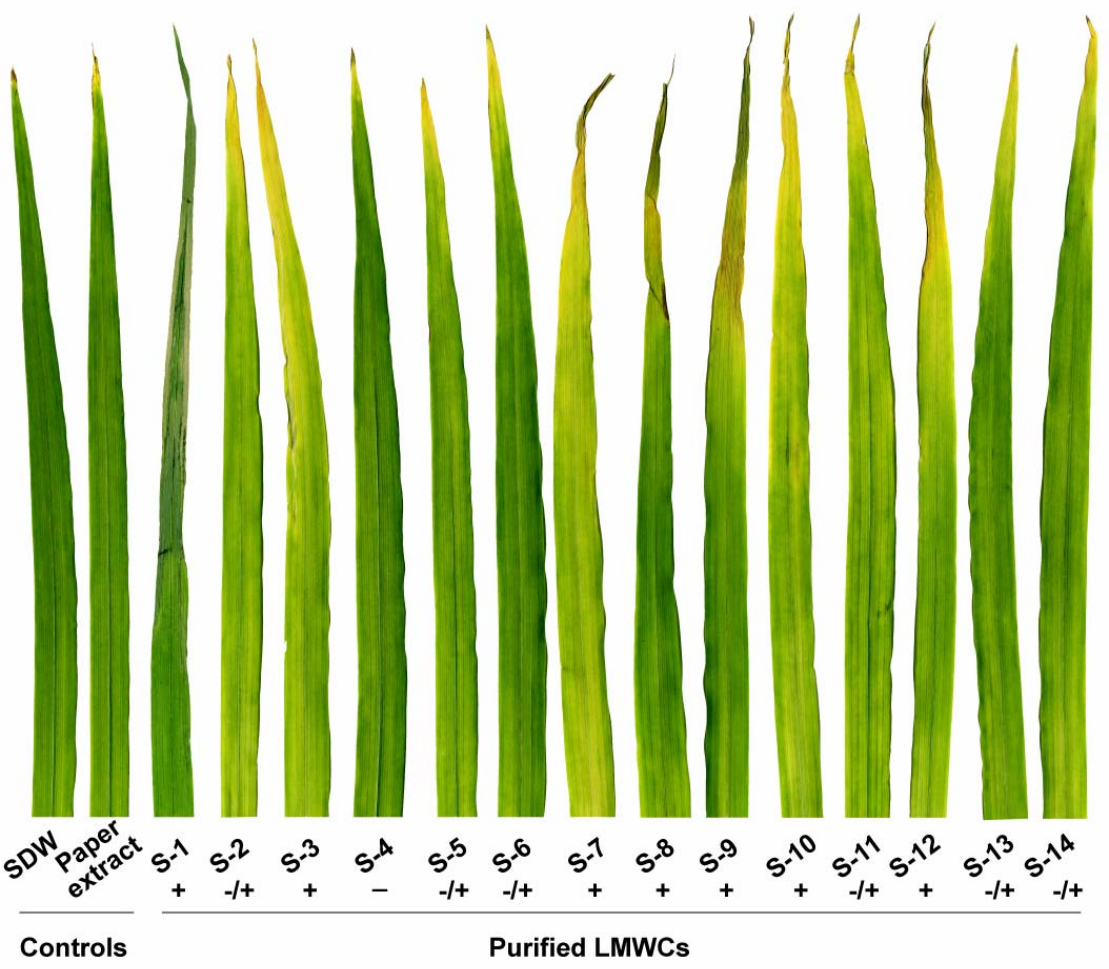


Figure 3.9 Biological activity of purified LMWCs extracted from culture filtrates of *Pyrenophora teres* f. *maculata* (*Ptm*) grown in PFCM. Each individual compound was purified using HVPE (see Figure 3.8) and 150 µL was used to treat excised barley leaves as per section 3.2.7. Treated leaves were monitored every 24 h, collected 144 h after treatment and classified as (+) if symptoms were present, (-/+) if weak symptoms were observed and (-) if no symptoms were evident. The leaves are representative of three separate experiments and were photographed 144 h post treatment except leaves treated with S-1 which were photographed after 72 h.

3.3.8 Tentative identification of LMWCs

The masses and electrophoretic properties of purified LMWCs were consistent with marasmines or amide linkage to marasmines (Table 3.1). Both S-1 and S-2 showed an M+H mass of 279.2 that is consistent with aspergillomarasmine B where the molecular weight is 278. However, the expected M+Na and M+H-18 masses of 301.2 and 261.2 were not observed (Figure 3.10). The other strong signal (391.6) with M+Na of 413.0 observed for both S-1 and S-2 may suggest an amide linkage of isoleucine to aspergillomarasmine B.

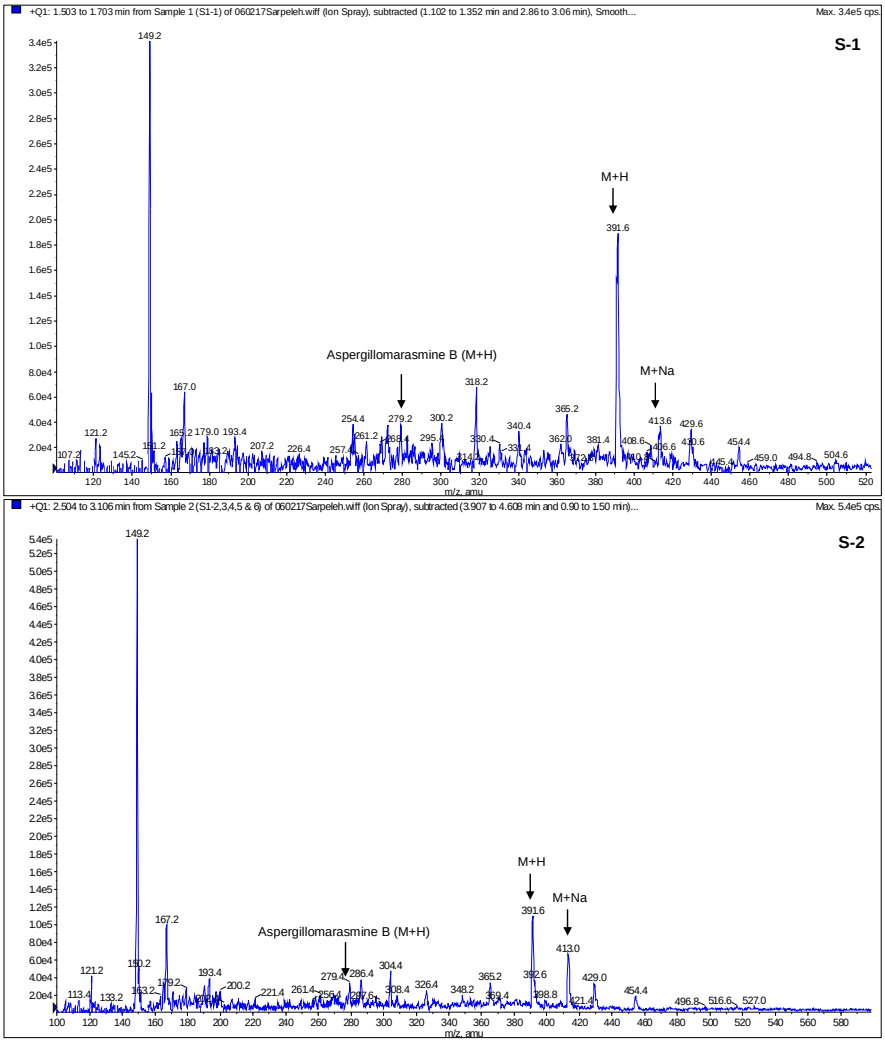


Figure 3.10 Mass spectra of S-1 and S-2. Each sample was purified using high voltage paper electrophoresis (HVPE) and the mass spectrum measured at conditions as per section 3.2.8 .

Table 3.1 Electrophoretogram properties and major mass spectral ions of purified LMWCs isolated from culture filtrates of *P. teres*. The electrophoretic mobility (R_m) of each compound is indicated relative to aspartic acid. Ninhydrin reaction and ultra violet (UV) absorbance was examined as per section 3.2.6. Ion spray mass spectrum conducted to detect the mass of each compound specified in the table as; one proton higher than the molecular weight of each individual compound (M+H), Molecular weight of each compound plus sodium mass (M+Na), and one H_2O mass less than M+H (M+H-H_2O). *: No corresponding mass detected in the mass spectrum data. ?: unknown compound.

Sample	R_m	Dye	UV	M+H	M+Na	M+H-H_2O	Putative compound
S-1	0.48	+	-	279.2	301.2	261.2	Aspergillomarasmine B
S-1	0.48	+	-	391.6	413.6	*	Isoleucine- aspergillomarasmine B?
S-2	0.57	+	-	279.2	301.2	261.2	Aspergillomarasmine B
S-2	0.57	+	-	391.6	413.6	*	Isoleucine- aspergillomarasmine B?
S-3	0.65	+	-	278.4	300.4	*	Lycomarasmine
S-3	0.65	+	-	348.2	370.2	330.4	?
S-3	0.65	+	-	308.2	330.4	*	Aspergillomarasmine A (Toxin C)
S-4	0.69	+	-	365.2	*	347.4	?
S-5	1.05	+	-	365.2	*	347.2	?
S-6	1.09	+	-	365.2	*	347.2	?
S-7	0.65	-	+	369.2	391.2	351.2	Conjugation of α keto acid of phenyl alanine to Toxin A (putative)
S-8	0.67	+	-	221.2	243.2	203.2	Toxin A
S-9	0.72	+	-	308.4	330.2	290.2	Aspergillomarasmine A (Toxin C)
S-9	0.72	+	-	290.2	312.2	272.4	Anhydroaspergillomarasmine A (Toxin B)
S-10	0.92	+	-	290.2	312.2	272.4	Anhydroaspergillomarasmine A (Toxin B)
S-11	1.05	+	-	365.2	*	*	?
S-12	0.82	+	-	290.2	312.2	272.2	Anhydroaspergillomarasmine A (Toxin B)
S-13	1.03	+	-	178.0	200.0	*	?
S-13	1.03	+	-	294.2	316.2	276.2	?
S-13	1.03	+	-	355.2	377.2	*	?
S-14	1.14	+	-	365.2	387.2	*	?
S-14	1.14	+	-	301.4	323.4	283.4	?

The mass spectrum detected for S-8 was consistent with *N*-(2-amino-2-carboxyethyl) aspartic acid (Toxin A) (Molecular weight=220) which showed a strong mass at 221.2 (M+H) with M+Na at 243.4 (Figure 3.11). The strong signal at 330.4 can be the M+Na for aspergillomarasmine A where the molecular weight is 307. However, other masses (M+H and M+H-H_2O) were not detected for this compound. The other masses observed at 369.2 (M+H) and 391.4 (M+Na) could be due to a reductive conjugation of an α keto acid of phenylalanine with Toxin A or traces of contamination between S-8 and S-7, where the two compounds ran very closly in the HVPE. The other mass at 370.2 was not consistent with the masses of the metabolites extracted from *P. teres* to date.

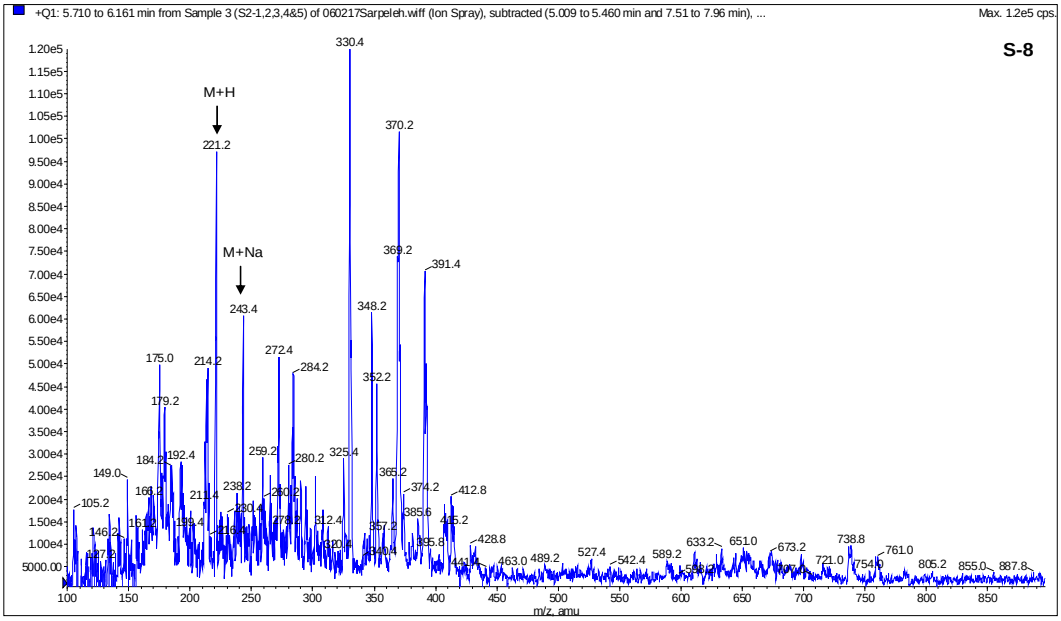


Figure 3.11 Mass spectrum of S-8. The sample was purified using high voltage paper electrophoresis (HVPE) and the mass spectrum measured at conditions described as per section 3.2.8.

S3 and S-9 (R_{m4}) showed one mass at 308.4 (M+H) with a mass at 330.2 (M+Na) which is consistent with aspergillomarasmine A (Toxin C). S-9 also showed another mass at 290.2 with M+Na at 312.4 which is consistent with

anhydroaspergillomarasmine A (Figure 3.12). S-3 (an artefact of S-1 and S-2) also showed a definite mass at 348.2 with M+Na at 370.2 which was not consistent with any compound isolated previously from *P. teres.* This compound also showed a weak mass at 278.4 which is consistent with the M+H ion for lycomarasmine (molecular weight = 277) (Figure 3.12).

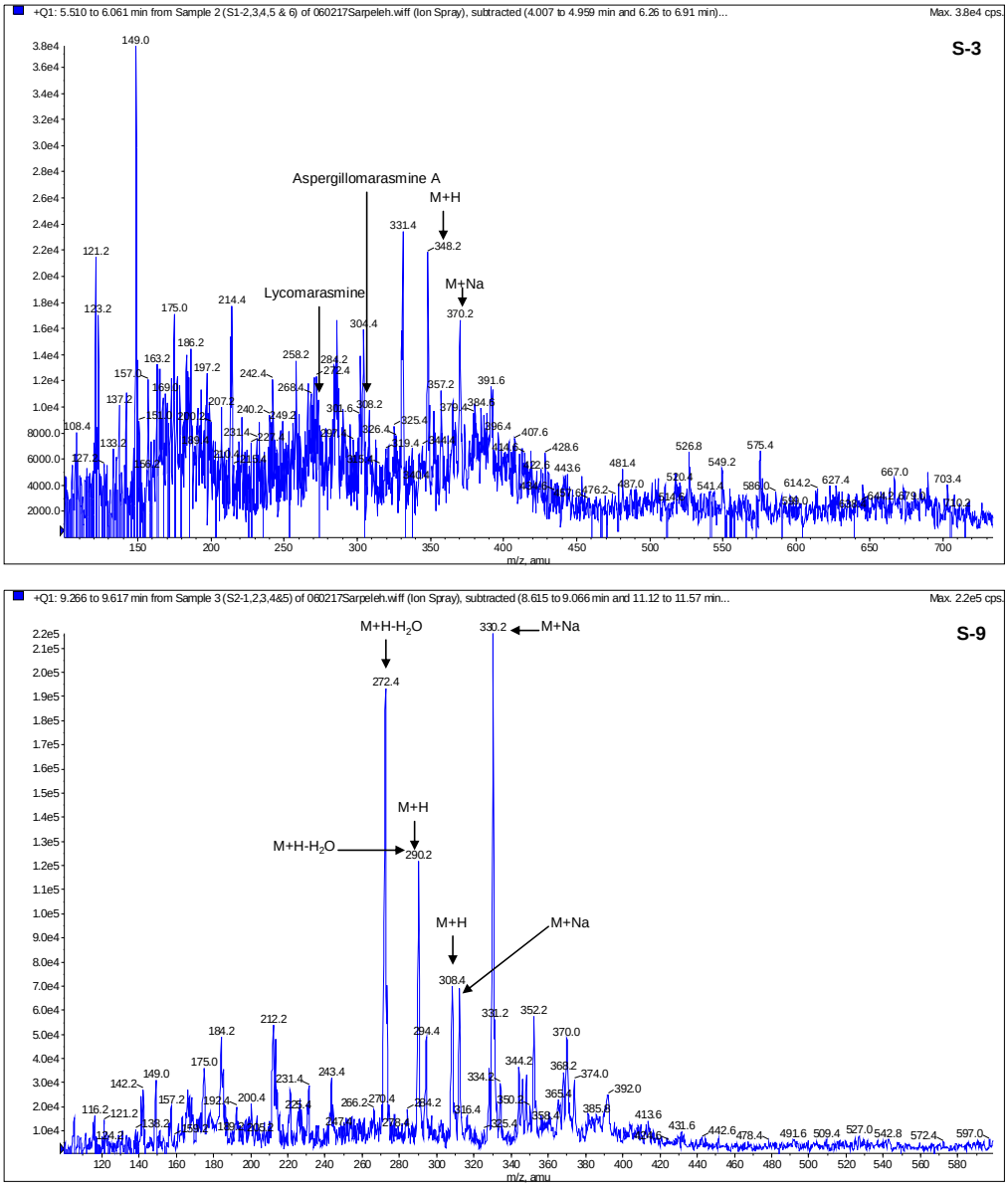


Figure 3.12 Mass spectra of S-3 and S-9. Each sample was purified using high voltage paper electrophoresis (HVPE) and the mass spectrum measured at conditions described as per section 3.2.8.

S-10 and S-12 comprised a definite mass at 290.2 (M+H) with the M+Na ion at 312.2 and M+H-H2O ion at 272.4 (Figures 3.13). All of these masses are consistent with anhydroaspergillomarasmine A (Toxin B) with a molecular weight of 289.

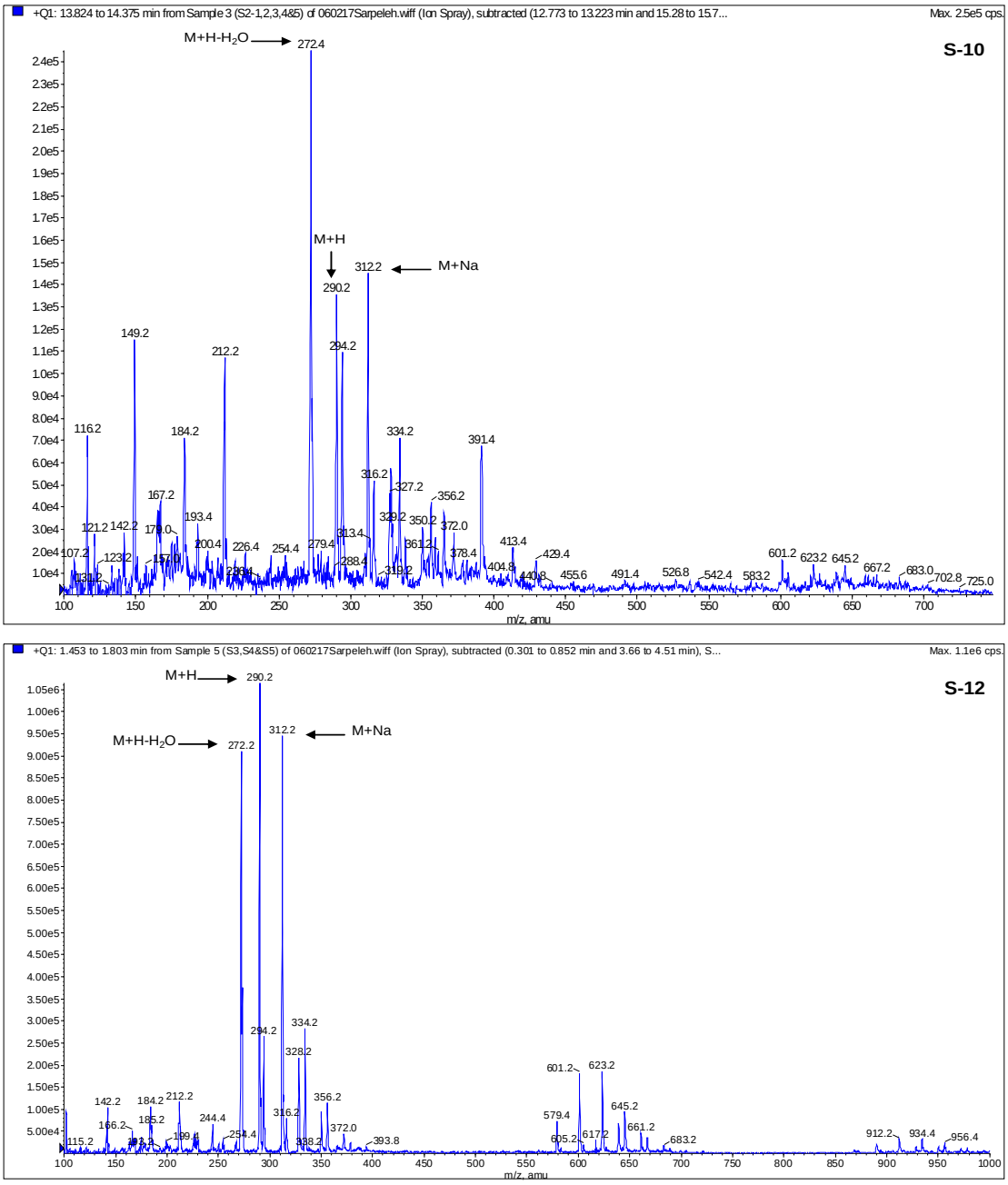
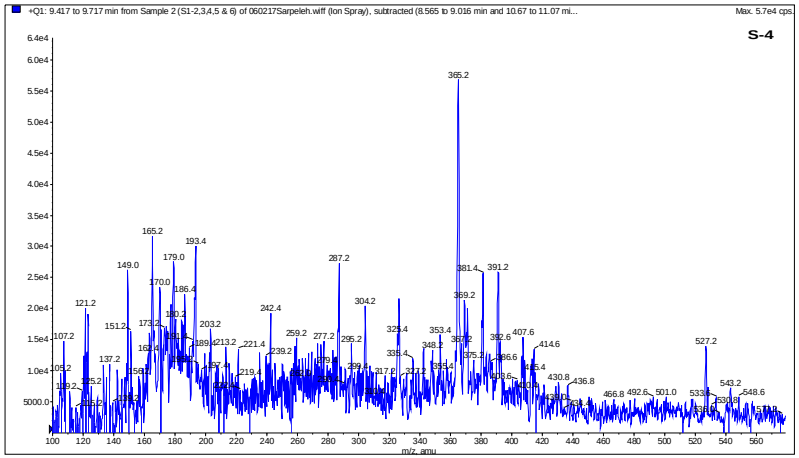


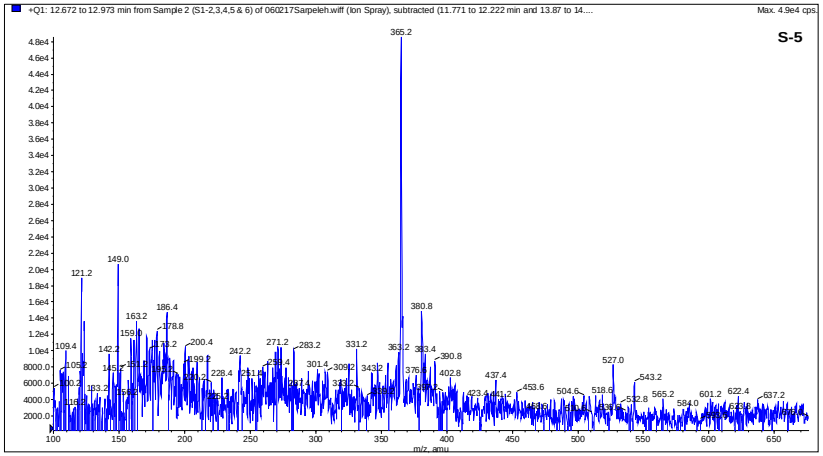
Figure 3.13 Mass spectra of S-10 and S-12. Each sample was purified using high voltage paper electrophoresis (HVPE) and the mass spectrum measured at conditions described as per section 3.2.8.

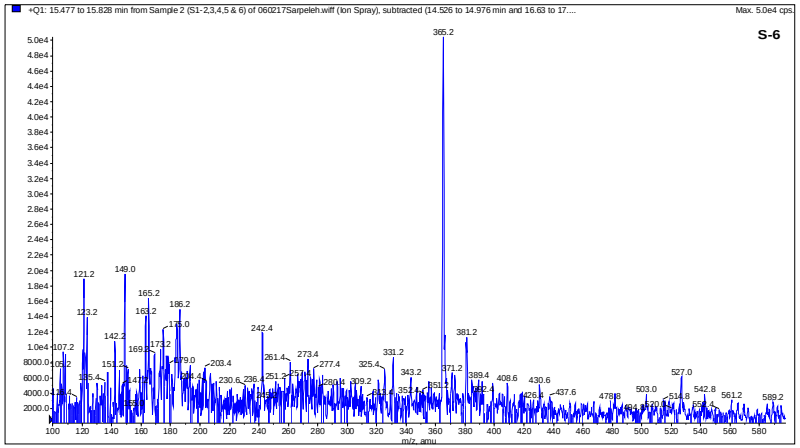
A strong mass was detected in S-4, S-5, S-6 and S-11 compounds at 365.2 (Figure 3.14) which showed no consistency with previously isolated compounds from *P. teres*. In addition, no masses of M+Na or M+H-H_2O were detected for these compounds. These compounds produced during the purification process (section 3.2.5), showed either no or only weak biological activity on treated barley leaves (Figure 3.9) and were considered as artefacts.

The masses detected for S-13 and S-14 also showed no consistency with previously isolated compounds from *P. teres* (Figure 3.15). These compounds only showed weak biological activity and therefore they were not further characterised.

S-7 which ran very close to S-8 (putatively identified as Toxin A) (Figures 3.3B and 3.8) showed no resemblance to previously isolated compounds from *P. teres* but had strong biological activity (Figure 3.9). This study therefore, considered this compound to be novel for this fungus and worthy of further characterisation. Unlike other LMWCs isolated in this study which turned purple, S-7 turned pink to red in reaction with ninhydrin (Figure 3.3B and 3.8). Additionally, this compound absorbed UV with its photospectrum similar to phenylalanine with one concentration-dependent peak (which is consistent with general absorbance) and one at 271.36 nm (Figure 3.16 A and B). Moreover, when S-7 was hydrolysed using hydrochloric acid, two ninhydrin-positive compounds were observed at the same mobility as phenylalanine and diaminopropionic acid (DAP). An unknown (?) ninhydrin-positive spot was also detected which was not consistent with any of the ninhydrin-positive species used in this study (Figure 3.16 C). Further, a strong signal at 369.2 (M+H) with M+Na at 391.2 (Figure 3.16 D) was detected in this compound which is consistent with a reductive conjugation of the α keto acid of phenylalanine to Toxin A.







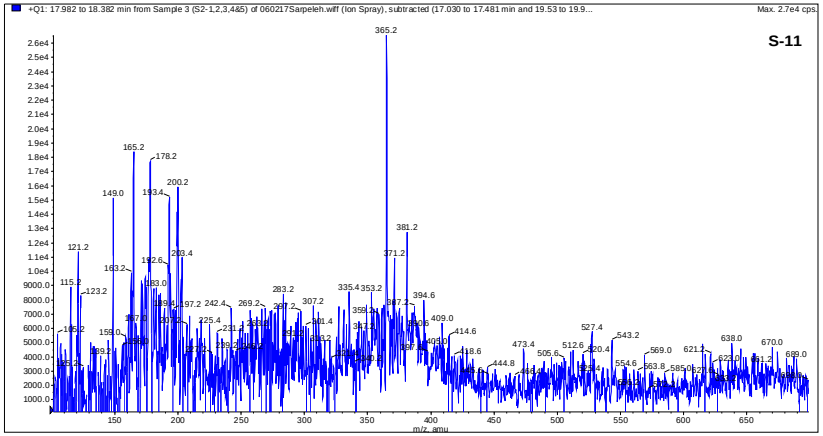


Figure 3.14 Mass spectra of S-4, S-5, S-6 and S-11. These samples were apparently produced during the purification process as artefacts and did not show any similarity to previously isolated compounds from *P. teres*.

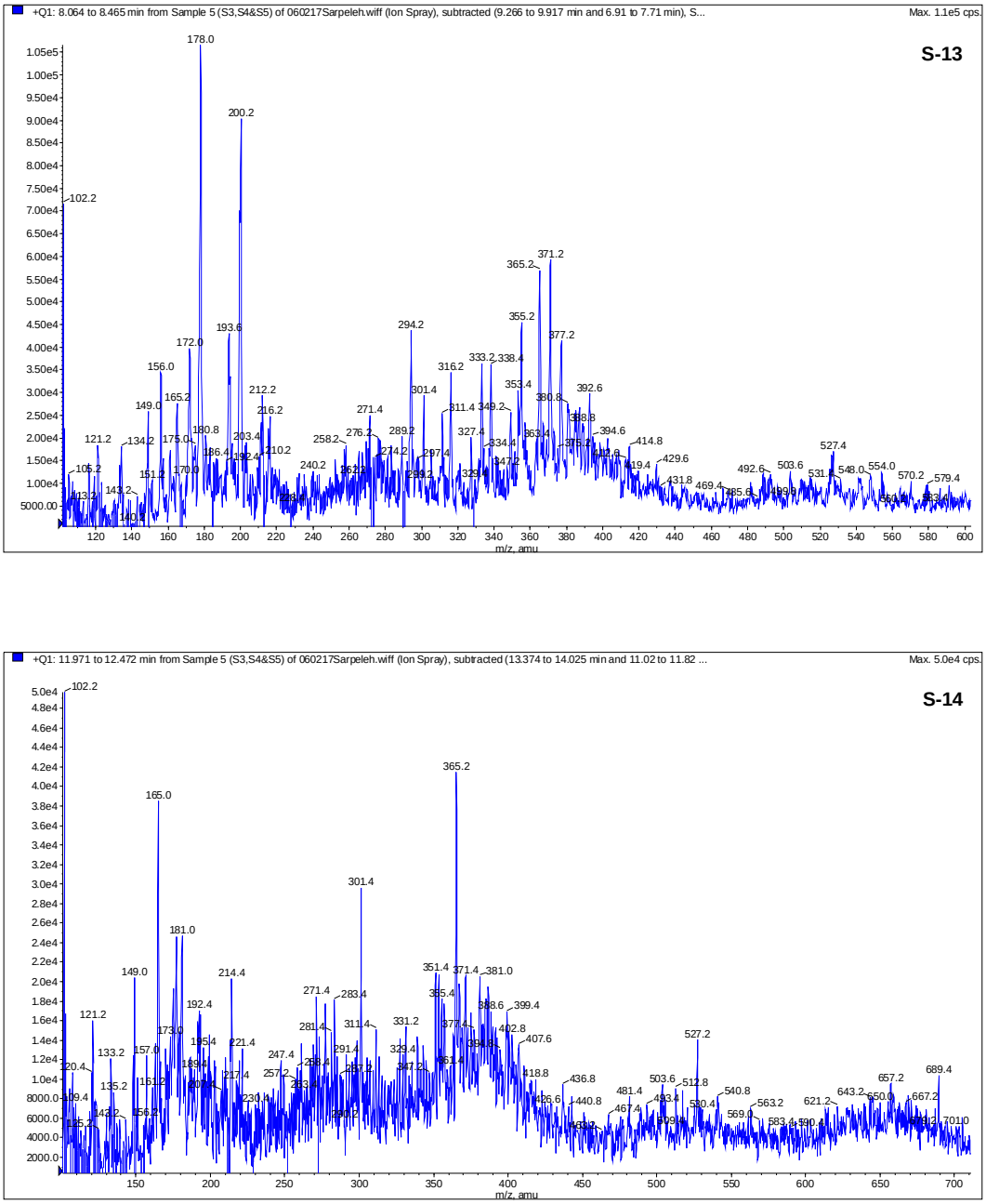


Figure 3.15 Mass spectra of S-13 and S-14. Each sample was purified using high voltage paper electrophoresis (HVPE) and the mass spectrum measured at conditions described as per section 3.2.8.

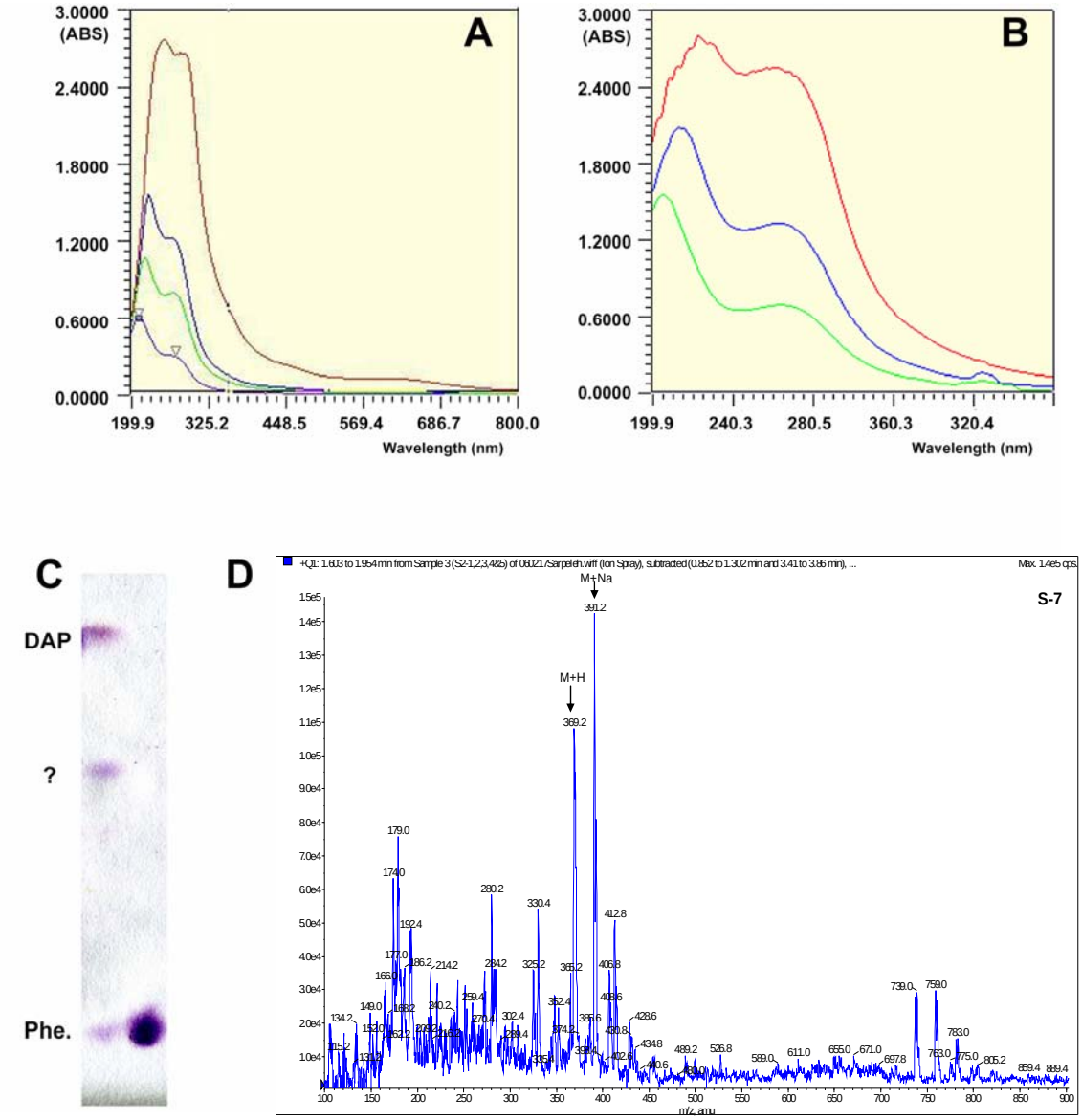


Figure 3.16 Putative identification of S-7. Four concentrations of phenylalanine (6.25, 12.5. 25, and 100 mM) gave a photospectrum (A) which was comparable to the S-7 photospectrum (B). Three Ninhydrin-positive compounds resulted from acid hydrolysis of S-7 as detected using HVPE (C). The mass spectrum of purified S-7 (D) was measured at conditions described in section 3.2.8. Phe: Phenylalanine, DAP: Diaminopropionic acid.

3.3.9 Chemical synthesis of Toxin A

The Toxin A synthesis reported by Friis et al. (1991) appeared to be sub-optimal, and was shown to be affected by the temperature regime, pH and incubation time. The reaction mixture of DL-DAP and maleic acid (in distilled H_2O at pH 6) incubated for 5h at 60 °C produced a single ninhydrin-positive compound (potentially Toxin A). A second compound at the same mobility as aspartic acid (R_m=1.0) was detected when the incubation time was increased to 15 h (data not shown). At 60 °C in buffer (pH 7.7 and 8.04) this compound (R_m=1.0) was not evident after this period of time (Figure 3.17A).

Incubation of the reaction mixture in buffer at 80, 100 and 120 °C ensured most of the DL-DAP was reacted with maleic acid by 15, 10 and 5h after incubation respectively. However, four ninhydrin positive compounds were detected in these thermal conditions suggesting degradation of the main product (Figure 3.17 B, C and D).

Incubation of D and L-DAP with maleic acid at 60 and 70 °C showed the same result as obtained for DL-DAP where increasing either the temperature or the incubation time resulted in the completion of the reaction but decomposition of the potential Toxin A into aspartic acid (Figure 3.18). Toxin A production was therefore trialled at 57 °C for 27 h and results suggest this would be optimal with no decomposition of Toxin A (Figure 3.19).

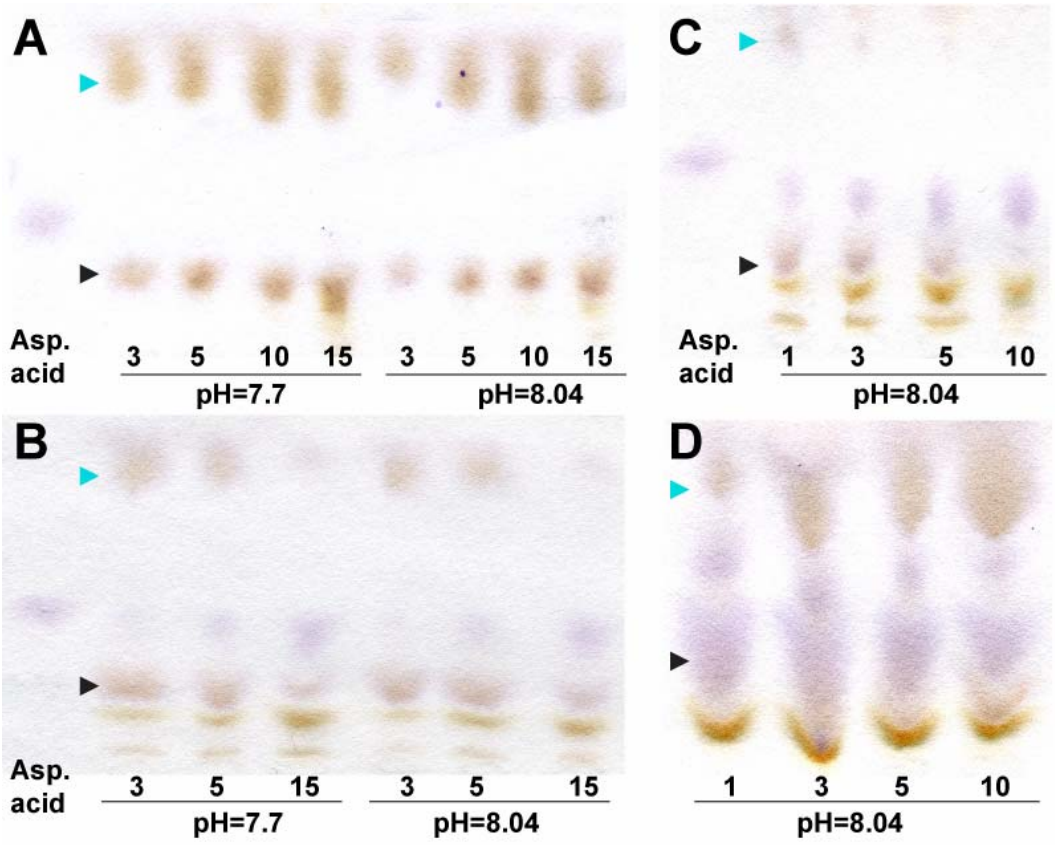


Figure 3.17 Effects of temperature and incubation time on Toxin A synthesis. Toxin A (►) was synthesised using maleic acid and DL-diaminopropionic acid (►) at 60 (A), 80 (B), 100 (C) and 120 °C (D) for 3, 5, 10 and 15 h incubation in buffered conditions (pH 7.7 and 8.04) and detected in the reaction mixture by high voltage paper electrophoresis as described in section 2.2.8.

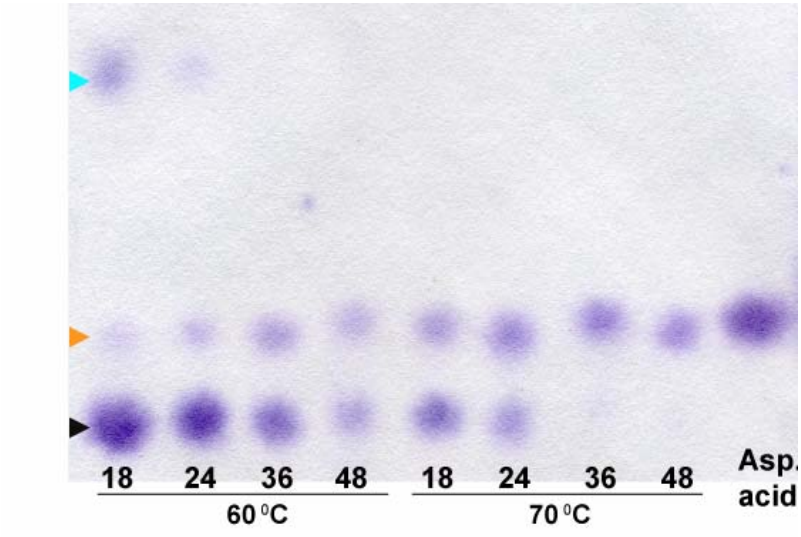


Figure 3.18 Effects of temperature and incubation time on Toxin A synthesis. Toxin A (►) was synthesised using maleic acid and L-diaminopropionic acid (L-DAP, ►) at 60 and 70 °C for up to 48 h incubation and detected in the reaction mixture by high voltage paper electrophoresis at pH 1.7 as described in section 2.2.8. An increase in temperature or time of incubation caused decomposition of Toxin A into aspartic acid (Asp. acid, ►). The results were similar in two independent experiments.

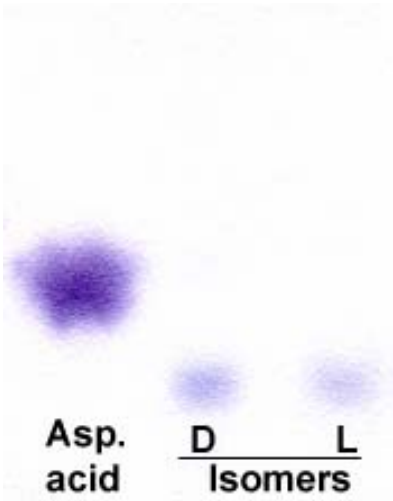


Figure 3.19 High voltage paper electrophoresis of D and L isomers of Toxin A. The isomers were synthesised using D and L-diaminopropionic acid and maleic acid at 57 °C for 27h and purified as described in section 3.2.9.2. Aspartic acid (Asp. acid) was run as a control.

3.3.10 Characterisation of synthesised Toxin A

Mass spectrometry of the synthesised compound using D and L-DAP confirmed the product was Toxin A since the D- isomer showed a mass at 221.07 with M+Na at 243.13 and M+H – H_2O at 203.07 (data not shown). The L-isomer showed similar masses at 221.13 (M+H), 243.13 (M+Na) and 203.13 (M+H-H_2O). When the product with a mass of 221 (M+H) was subjected to MS/MS, the same profile was observed suggesting that they were isomers of the same compound (Figures 3.20).

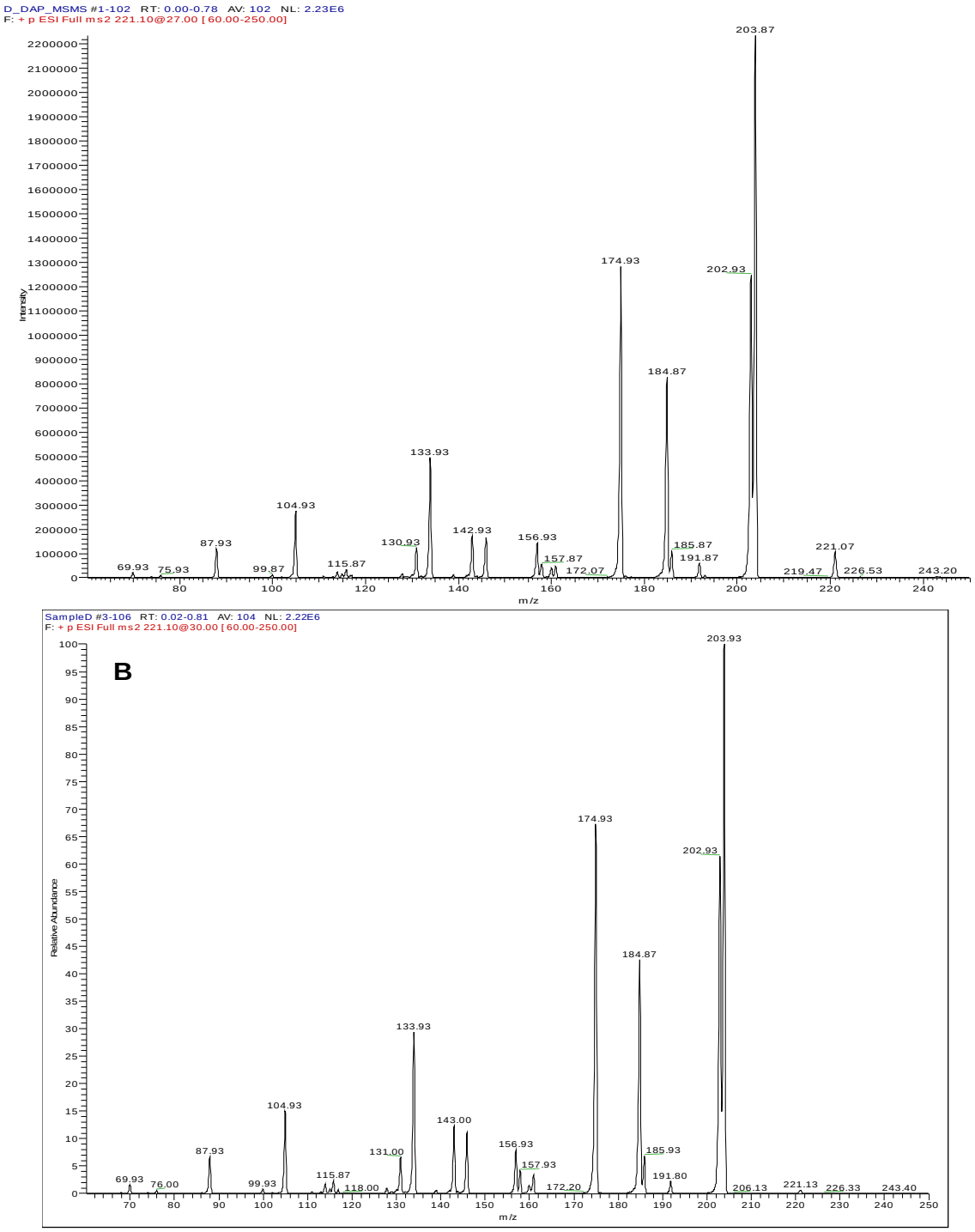


Figure 3.20 Characterisation of synthesised Toxin A using MS/MS. D and L isomers of Toxin A were synthesised and their mass spectra were performed as described in section 3.2.9.3. The Ms/Ms data of the ion at 221 (related to Toxin A) for D isomer (A) and L-isomer (B) was conducted as described in section 3.2.9.3.

3.3.11 Biological activity of synthesised Toxin A

The synthetic mixture of diastereoisomeric racemates (resulting from the reaction between DL-DAP and maleic acid), as well as individual D and L isomers of Toxin A synthesised in this study, showed biological activity when used to treat excised barley leaves (Figures 3.21 and 3.22). The symptoms induced by the mixture of isomers synthesised using DL-DAP was expressed as water soaking in the tips of the leaves as early as 6 h post treatment followed by chlorosis and general necrosis 36 to 48 h after treatment (Figure 3.21).

There was no difference between D and L isomers in symptom induction with both inducing water soaking first followed by extensive chlorosis which started from the tips of the leaves and progressed downwards. Water soaking was observed as soon as 12 h after treatment with 100 μM of both isomers while water soaking was observed 24 h after treatment with 50 μM of the toxins (data not shown). Similarly, chlorosis was observed 72 h after treatment of the leaves with higher concentrations of the toxins and at 96 h for those treated with 50 μM of phytotoxins (Figure 3.22).

The symptoms expressed by the racemic mixture of isomers appears to be stronger than those induced by D or L isomers where the base of the leaves which were in contact with the mixture of diastereoisomeric racemates showed necrosis 24 h post treatment. However, the presence of other by-products (see Figure 3.17) suggests the presence of other bioactive compounds affecting the biological activity. Control plants treated with sterile distilled water (SDW) and SDW containing 0.1 % ammonia did not show any symptoms (Figures 3.21 and 3.22).

Figure 3.21 Biological activity of a synthetic mixture of diastereoisomeric racemates of Toxin A (potential D, L, DL and LD isomers). Excised barley leaves (Sloop) were treated with different concentrations of a mixture of isomers of Toxin A (synthesised using DL-diaminopropionic acid and maleic acid at 80 °C as described in section 3.2.9.4. Control leaves were treated with sterile distilled water (SDW) and SDW containing 0.1 % ammonia. The samples were collected after 120 h and are representative of six leaves treated in two independent experiments.

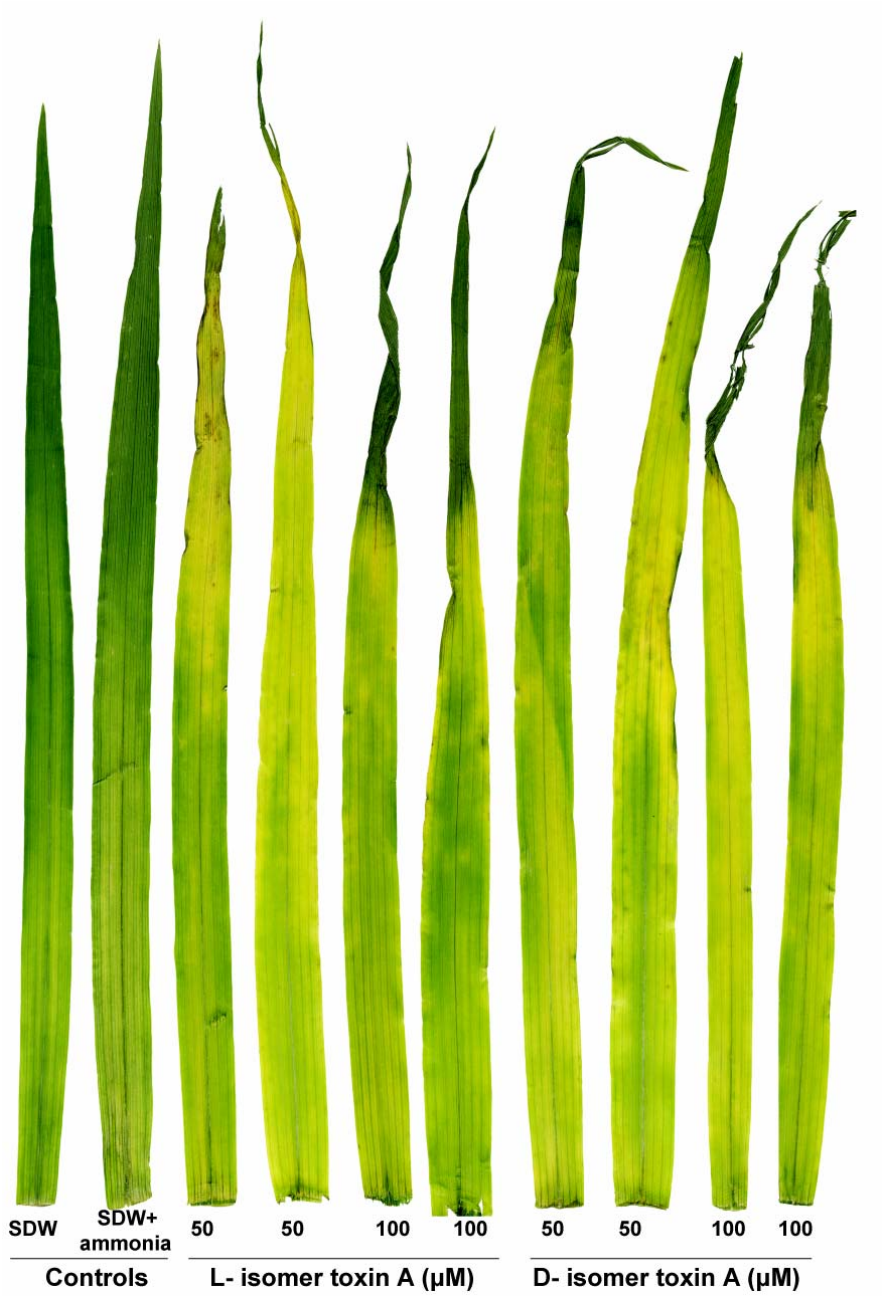


Figure 3.22 Biological activity of synthetic isomers of Toxin A. D and L isomers of Toxin A were synthesised as described in the text and used to treat excised barley leaves (Sloop) at two different concentrations (50 or 100 µM). Control leaves were treated with sterile distilled water (SDW) and SDW containing 0.1 % ammonia. The leaves were collected 120 h post treatment and are representative of six leaves treated in two independent experiments.

3.3.12 Chemical synthesis of S-7

S-7 was synthesised using the synthesised Toxin A (L-isomer) and sodium phenyl pyruvate as described earlier (section 3.2.9.5). S-7 was observed in the reaction mixture by HVPE where a pink to reddish spot was obvious in reaction with ninhydrin. Additionally, two other compounds were detected in the reaction mixture; one UV absorbing compound at zero mobility which was the excess of sodium phenyl pyruvate and one ninhydrin positive compound which was the excess of Toxin A (Figure 3.23). Further purification and characterisation of the compound was not carried out due to the time constraints.

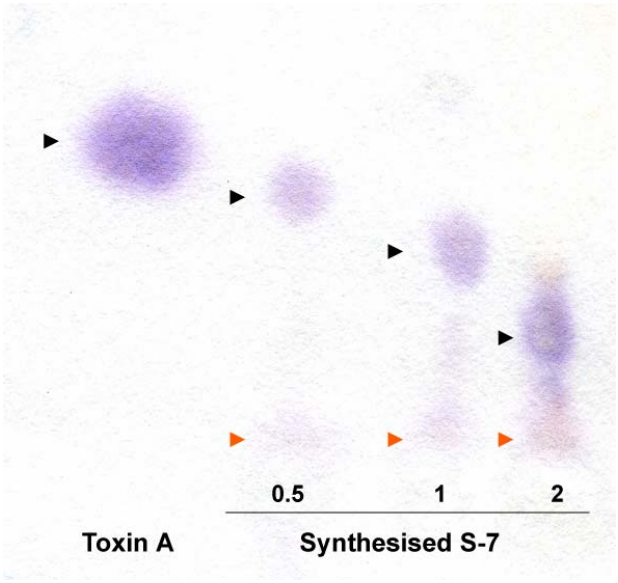


Figure 3.23 Potential S-7 synthesised compound. S-7 (▶) was synthesised using the L-isomer of Toxin A (▶) and sodium phenyl pyruvate and visualised by HVPE in reaction with ninhydrin as described in section 3.2.9.5. Two µL of Toxin A (as control) and different amounts of the product (S-7) were loaded in HVPE. The picture is representative of two independent experiments. The decrease in the mobility of Toxin A with the increase loading is due to the increased concentration of salts in the reaction mixture.

3.4 Discussion

In this study, the electrophoretic properties and staining of the toxins indicated that both forms of *P. teres* produced similar toxins in the conditions grown. Similarly, previous investigation of the toxin constituents of *P. teres* did not find any differences between the toxin contents of the two forms of the fungus (Smedegaard-Petersen 1977b, Weiergang *et al.* 2002b). As a consequence, the toxins purified and characterised here were isolated from the spot form of the pathogen (*Ptm*) which produced higher amounts of toxins compared with the net form (*Ptt*) in the same growing conditions.

Studies on the *in vitro* production of toxins indicate that isolates of *P. teres* are prospective producers of several toxins which belong to different chemical classes including marasmines, pyrenolides and pyrenolines (Smedegaard-Petersen 1977b, Bach *et al*. 1979, Friis *et al*. 1991, Coval *et al.*1990, Nukina *et al.* 1980, Nukina *et al.* 1980, Nukina and Hirota 1992, Weiergang *et al*. 2002b). However, *P. teres* produces different types or quantities of LMWCs under different growing conditions (Weiergang *et al.* 2002b) as do other plant pathogens (Alam *et al.* 1989, Beed *et al.*1994, Chen and Strange 1991, Jenns *et al.* 1989, Robenson and Strobel 1986, Tegli *et al.* 1994). In the present study, only marasmines or related derivatives were isolated but not pyrenolides or pyrenolines which have previously been obtained from different strains of *P. teres* using different culture media and growing conditions (Coval *et al.* 1990, Nukina and Hirota 1992, Nukina *et al.* 1980). Unlike the known marasmines which are ninhydrin-positive metabolites, pyrenolines and pyrenolides are UV absorbing metabolites and readily visualised under UV light as bright blue bands or spots (Coval *et al.* 1990, Nukina and Hirota 1992, Nukina *et al.* 1980). With the exception of S-7, none of our compounds showed UV absorbance, and no masses

of phytotoxic species were consistent with pyrenolines or pyrenolides, indicating the metabolites isolated in this study have no resemblance with pyrenolines or pyrenolides.

In the present study, eight LMWCs were isolated from *P. teres* observed as ninhydrin-positive metabolites in which two compounds, S-7 and S-8, were nearly over-lapping with R_{ms} 0.65 and 0.67 respectively (Figure 3.3 and 3.6). Additionally, some metabolites were produced as artefacts during the purification processes with no or weak phytotoxicity. For example, six ninhydrin positive spots were identified from the second run of the first two metabolites (S-1, R_{m1} 0.48 and S-2, R_{m2} 0.57) (Figures 3.3 and 3.7) in which only two compounds were consistent in relative mobility with those extracted in the first run (S-1 and S-2). The other compounds (S-3, S-4, S-5 and S-6) are assumed to be artefacts which arose during extraction, freeze drying and re-running on the papers. The same situation can be postulated for S-10 and S-11 which arose after further purification of the toxic metabolites (S-7, S-8 and S-9). Previous work on toxins produced by *P. teres* showed non-enzymatic conversion of aspergillomarasmine A (Toxin C) into anhydroaspergillomarasmine A (Toxin B) at low pH (Friis *et al*. 1991) and this is probably the case for conversion of S-9 (aspergillomarasmine A) to S-10 (anhydroaspergillomarasmine A).

The other supposed artefacts isolated in this study (S-3, S-4, S-5, S-6 and S-11) may have arisen via weak acid hydrolysis during the purification process as reported in previous research (Smedegaard-Petersen 1977b) wherein acid hydrolysis of toxins A and B produced five and six metabolites respectively. Alternatively, ninhydrin-positive compounds (S-4, S-5, S-6 and S-11) which showed a strong mass at 365.4 (M+H) can be postulated as *N*-acyl peptide linkages of glycine residue (=57) to

aspergillomarasmine A (MW=307). Other masses related to these compounds can be interpreted as serine linkages to lycomarasmine due to the potential addition of a serine residue (= 87) to lycomarasmine (molecular weight=277) corresponds to the strong M+H mass at 365.2. Lycomarasmine has been previously isolated from liquid culture of *Fusarium oxysporum* f. sp. *lycopersici* together with another inactive compound named "Substance I" (Barbier 1972, Hardegger *et al*. 1963). Lycomarasmine was found to be toxic to *Solanum tuberosum*, *Pelargonium zonale* and *Vitis vinifera* (Bach *et al.* 1979). It is also toxic to *Gossipium herbaceum*, *Impatiens holstii*, *Lupinus polyphyllus*, *Phaseolus vulgaris*, *Pisum sativum* and *Ricinus comminis* when complexed with iron (Bach *et al.* 1979). In the present study, the lycomarasmine-like compounds showed no (S-4) or weak phytotoxicity (S-5, S-6 and S-11).

The combined chemical evidence (comprising mass spectrometry, electrophoretic mobilities and staining properties) demonstrated that S-1, S-8, S-9 and S-12 are consistent with the previously described compounds; aspergillomarasmine B, *N*-(2-amino-2-carboxyethyl) aspartic acid (Toxin A), aspergillomarasmine A (Toxin C) and anhydroaspergillomarasmine A (Toxin B) respectively. Since S-1 and S-2 showed the same masses with different relative mobilities and biological activity, they may indicate the presence of different isomers. For example, both compounds showed a strong signal at 279.2 that is consistent with M+H ion for aspergillomarasmine B (molecular weight = 278) and the addition of the residue of leucine or isoleucine both of which have residue masses of 113 corresponding to the strong M+H ion at 391.6 with the M+Na ion that is apparent at 413.6. All of this is consistent with an amide linkage of aspergillomarasmine B to either leucine or isoleucine. Alternatively, as there are four carboxyls in aspergillomarasmine B (Barbier 1972), there are four

possible locations for the amide linkage to either leucine or isoleucine that could represent the mass at 391.6. Aspergillomarasmine B has been previously isolated from cultures of *Aspergillus flavus oryzae* and *Colletotrichum gloeosporioides* (Ballio *et al.* 1969, Haenni *et al.* 1965). This compound has only been isolated in trace amounts from *P. teres* in this study and probably is involved in the infection of barley by the fungus. In the bioassay of barley leaves, aspergillomarasmine B (S-1) showed strong phytotoxicity including water soaking and general necrosis 24 and 48 h post treatment respectively, but not the extensive chlorosis which was induced by the other compounds isolated in this study. Marasmines can form chelates with iron ions occurring naturally in the plants or toxin solution and the iron chelates are more toxic to the plant than the pure toxin alone (Gaumann *et al.* 1950, Robert *et al.* 1962, Bach *et al.* 1979) due to the molecular presence of two ethylenediamine groups attached to carboxylic group. This provides a structure similar to the structure of ethylendiaminetetraaceticacid (EDTA) (Barbier 1983). The high phytotoxicity observed for aspergillomarasmine B, which is closely related structurally to lycomarasmine and aspergillomarasmine A (Figure 3.1), could be because of the formation of a toxic iron chelate inside the culture medium, especially ferric sulphate, which was a component of the culture medium.

The mass spectrum and the electrophoretic properties observed for S-8 are consistent with *N*-(2-amino-2-carboxyethyl) aspartic acid (Toxin A). Toxin A is proposed to be the direct precursor of aspergillomarasmine A (Friis *et al*. 1991). Aspergillomarasmine A was detected in S-9 which showed (Figure 3.12) a significant signal at 330.2 which is consistent with the M+Na for aspergillomarasmine A. Aspergillomarasmine A has been previously isolated from *P. teres* (Bach *et al.* 1979, Weiergang *et al.* 2002b) and other fungi (Barbier 1972, Haenni *et al.* 1965, Trouvelot

et al. 1971, Bousquet *et al.* 1971) and demonstrated to be the toxin which plays a major role in disease induction by *P. teres* in barley (Friis *et al.*1991).

Mass spectral and electrophoretic data confirmed the existence of anhydroaspergillomarasmine A (Toxin B) in this study where strong signals at 290.2 (M+Na=312.2 and M+H - H_2O=272.2) were detected in S-12, which is consistent with Toxin B. Several studies have isolated this toxin as the major compound from *P. teres* (Smedegaard-Petersen1977b, Bach *et al.* 1979, Friis *et al.* 1991, Weiergang *et al*. 2002b). However, the toxin was proposed as an artefact of aspergillomarasmine A dehydration (Barbier 1972, Bach *et al*. 1979), which the latter transformed to Toxin B due to a non-enzymatic conversion under a low pH of the culture medium (Friis *et al.* 1991) or treatment with triflouracetic acid (Bach *et al*. 1979). Similarly, Toxin B was found in higher amounts compared with either Toxin A or Toxin C in this study. Although the pH of the culture medium employed in the present study was nearly constant with minor changes between 6.8 and 6.17, the isolation of Toxin B might be due to the type of isolates used in this study since, individual isolates of *P. teres* were shown to be variable in their ability to produce toxin B or C under different culture and incubation conditions (Weiergang *et al.* 2002b).

The simple structure of Toxin A provides a ready synthesis under the modified conditions reported in this work. Additionally, the structure of this toxin (Figure 3.1) indicates it could be the precursor or a component of some of the other metabolites isolated in this study such as S-7. Four possible stereoisomers of Toxin A have been previously synthesized using maleic acid and diaminopropionic acid (Friis *et al*. 1991) in which LL-isomer was shown to be the most bioactive isomer of the toxin. This study did not find such a difference between the different isomers of Toxin A where L

and D isomers showed similar activity while the mixture of diastereoisomeric racemates synthesised using DL-DAP showed apparently stronger biological activity in treated barley leaves. One possibility is that all isomers are converted to some other toxic product prior to an effect becoming evident in the bioassay. The differing toxicities observed by Friis et al. (1991) could reflect different races of conversion to this hypothetical ultimate toxin. One possible candidate could be the inter-conversion of the D, L and the DL 2-amino Toxin A molecules via either a non-selective transaminase or (D and L-) transminases into the corresponding 2-keto-Toxin A which then acts as a keto-acid acceptor for the alpha amino group of some amino acids or peptides (personal communication, Max E. Tate).

S-7 showed no resemblance with previously isolated compounds from *P. teres* and suggested to be a reductive conjugation of a potential α-keto acid of phenylalanine to Toxin A. The UV absorbance at 270 nm suggested the presence of an aromatic structure in the molecule. Acid hydrolysis of S-7 revealed the presence of a compound at the same mobility as phenylalanine (Figure 3.16C). Additionally, crude filtrates of the culture medium showed several neutral amino acids including an amino acid of the same mobility as phenylalanine (data not shown). Further, the masses detected for this compound suggested the presence of phenylalanine in its structure and as a consequence, S-7 may have been directly excreted by the fungus into the culture medium or converted by post-modification of Toxin A through an imino linkage to this compound via the amino group available in the Toxin A (Figure 3.1). This interpretation provided the opportunity to synthesise S-7 chemically, using sodium phenyl pyruvate and Toxin A in the presence of sodium cyanoborohydride (Figure 3.24). Preliminary experiments showed that a compound appeared in the reaction mixture which like S-7 appeared pink to reddish in reaction with ninhydrin.

However, further experiments need to be performed to optimise the synthesis and to further characterise this new compound from *P. teres*.

Although this study used the same protocol previously established to isolate LMWCs from *P. teres* culture filtrates (Smedegaard-Petersen 1977b, Friis *et al.* 1991, Weiergang *et al.* 2002b), the use of other strains of *P. teres* (section 2.2.2) rather than those used in previous research demonstrated the potential production of Toxin A and a number of its derivative including the newly identified conjugation of a potential α-keto acid of phenylalanine to Toxin A and its role in symptom development in net blotch disease of barley.

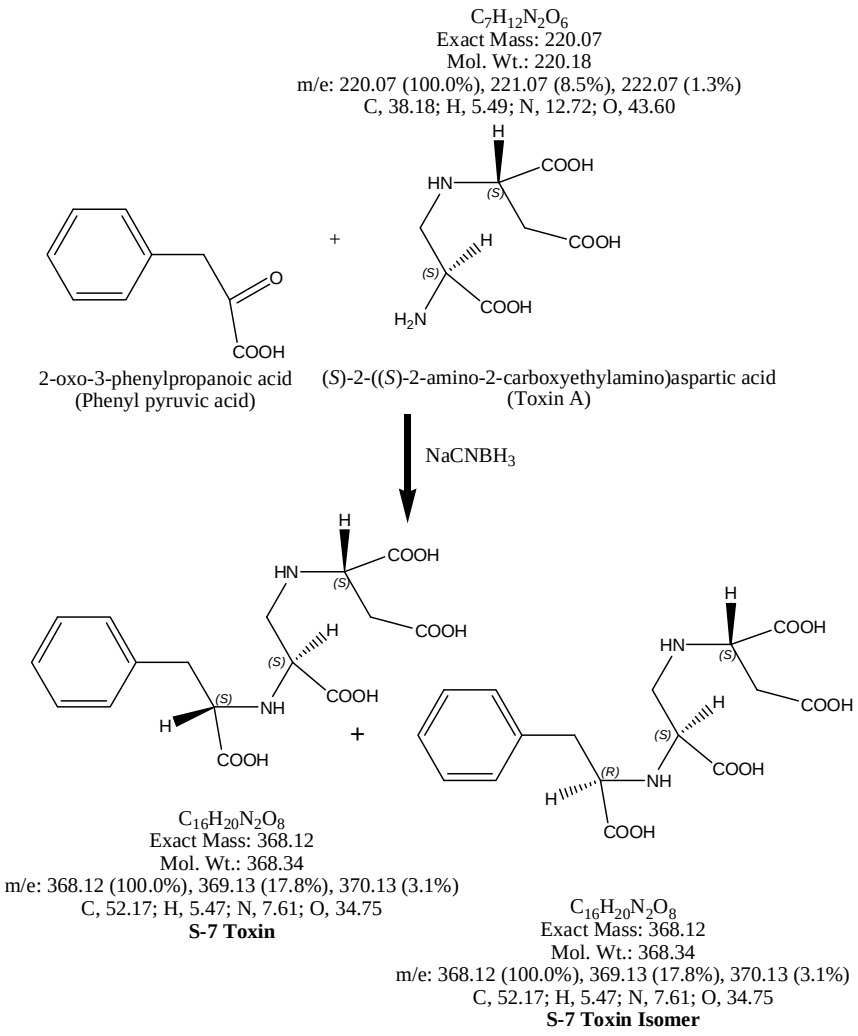


Figure 3.24 A method for synthesising S-7 from *P. teres* (a reductive conjugation of α-keto acid of phenylalanine to Toxin A). The two isomers of the compound can be produced using phenyl pyruvic acid and Toxin A in the presence of sodium cyanoborohydride as described above.