

PROTEOMIC RESPONSES OF UNINFECTED TISSUES OF PEA
PLANTS INFECTED BY ROOT-KNOT NEMATODE, *FUSARIUM*
AND DOWNY MILDEW PATHOGENS

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Abstract

Peas suffer from several diseases, and there is a need for accurate, rapid in-field diagnosis. This study used proteomics to investigate the response of pea plants to infection by the root knot nematode *Meloidogyne hapla*, the root rot fungus *Fusarium solani* and the downy mildew oomycete *Peronospora viciae*, and to identify potential biomarkers for diagnostic kits. A key step was to develop suitable protein extraction methods. For roots, the Amey method (Chuisseu Wandji *et al.*, 2007), was chosen as the best method. The protein content of roots from plants with shoot infections by *P. viciae* was less than from non-infected plants. Specific proteins that had decreased in abundance were (1->3)-beta-glucanase, alcohol dehydrogenase 1, isoflavone reductase, malate dehydrogenase, mitochondrial ATP synthase subunit alpha, eukaryotic translation inhibition factor, and superoxide dismutase. No proteins increased in abundance in the roots of infected plants. For extraction of proteins from leaves, the Giavalisco method (Giavalisco *et al.*, 2003) was best. The amount of protein in pea leaves decreased by age, and also following root infection by *F. solani* and *M. hapla* at six weeks post-inoculation. *F. solani* caused a decrease in abundance of isocitrate dehydrogenase, glycerate dehydrogenase, carbonic anhydrase, oxygen evolving enhancer protein 2 (OEE2), phosphoglycerate kinase, chloroplastic and one unknown protein. Some leaf proteins increased in abundance, and included heat shock-related proteins (HSP70) and two unknown proteins. Proteins that decreased in leaves following root infection by *M. hapla* six week post-inoculation were RuBisCo large subunit, fructose biphosphate aldolase 2, carbonic anhydrase, OEE1, OEE2, OEE3, RuBisCo small subunit and a 28KDa ribonucleoprotein. Some proteins increased in abundance, such as HSP70, fructose biphosphate aldolase 1 and trypsin. In contrast

to the decrease in protein observed at six weeks post-inoculation, the amount of protein increased in leaves three weeks after inoculation of roots with *M. hapla*.

Root infection by both *M. hapla* and *F. solani* caused a reduction in leaf area, and also a reduction in fresh and dry weight of the shoot and root systems.

The use of digital imaging and visible and infra-red light to study the changes in leaves was explored in this study. A clear difference was visible between leaves from healthy plants and between those from *M. hapla* and *F. solani* infected plants when imaged using a normal digital camera. In contrast, no clear differences were noticed between leaves of healthy, *M. hapla* and *F. solani* infected plants when using an infra-red camera with 850 nm wavelength light.

This study indicates that specific proteins are altered in abundance in leaves following root infection, and provides the basis for future studies to develop rapid diagnostic tests.

Declaration

I declare that the fulfilling of this thesis entitled proteomic responses of uninfected tissues of pea plants infected by root knot nematode, *Fusarium* and downy mildew pathogens, is a result of original research work, and that I acknowledge anyone who gave me any help during this work. I confirm that this work has not been submitted for a degree or as part of the requirement for a degree at any university or institution other than the University of the West of England.

Al-Sadek Ghazala

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Dedication

“To the spirits of my
departed Father and
Brother”.

Table of contents

Abstract	i
Declaration	iii
Acknowledgments.....	iv
Dedication	v
Table of contents	vi
List of figures	xiii
List of Tables.....	xxi
1. Introduction.....	1
1.1. Background	2
1.2. Pea diseases	4
1.2.1. Pea downy mildew	6
1.2.2. <i>Fusarium</i> root rot	10
1.2.3. Nematode diseases	14
1.3. Diagnosis of plant diseases.....	19
1.4. Plant disease diagnostics for field use	22
1.5. Biosensors for diagnosis of pea diseases.....	24
1.6. Proteomics	27
1.7. Early detection of pea root disease using infrared imaging	29

1.8. Estimation of leaf area, fresh and dry weight of pea shoot and root system from plants infected by <i>F. solani</i> f. sp. <i>pisi</i> and <i>M. hapla</i>	30
1.9. Plant disease control.....	31
1.10. Methods of disease control.....	31
1.11. Aims of the project.....	36
2. Materials and Methods.....	38
2.1. <i>Pisum sativum</i> L. (pea) cultivars.....	39
2.2. <i>Solanum lycopersicon</i> (tomato) cultivars.....	39
2.3. Inoculation of pea plants with <i>Peronospora viciae</i> f. sp. <i>pisi</i>	40
2.3.1. Leaf inoculation.....	40
2.4. Inoculation of pea plants with <i>Fusarium solani</i> f. sp. <i>pisi</i>	41
2.4.1. Soil inoculation method of Clarkson (1978).....	41
2.4.2. Seed inoculation method of Kraft and Kaiser (1993).....	42
2.5. Inoculation of pea plants with <i>Meloidogyne hapla</i>	42
2.6. Methods of protein extraction.....	43
2.6.1. Pea roots.....	43
2.6.1.1. Amey method (Chuisseu <i>et al.</i> , 2007).....	43
2.6.1.2. Brigham method (Brigham <i>et al.</i> , 1995).....	44
2.6.1.3. SDS and SDS-acetone methods (Shultz <i>et al.</i> , 2005).....	44
2.6.1.4. TCA-acetone method (Natarajan <i>et al.</i> , 2005).....	45
2.6.1.5. BPP method (Wang <i>et al.</i> , 2007).....	45
2.6.2. Pea leaves.....	46

2.6.2.1. Giavalisco method (Giavalisco <i>et al.</i> , 2003).....	46
2.6.2.2. Amey method (Chuisseau <i>et al.</i> , 2007).....	47
2.7. Conductivity	47
2.8. Protein desalting	48
2.8.1. 2D Clean-up Kit	48
2.8.2. Zeba De-salt Spin Column	49
2.9. Protein quantification (Bradford method)	49
2.10. 1D-gel electrophoresis (SDS PAGE)	50
2.11. 2D-gel electrophoresis.....	51
2.11.1. Isoelectric focusing	52
2.11.2. IPG-strip equilibration	53
2.11.3. Preparing the Ettan DALT six gel casters (GE Healthcare, Buckinghamshire, England).....	54
2.11.4. Unloading the gel caster.....	55
2.11.5. Second dimension	56
2.12. PDQuest Spot Analysis	58
2.13. Protein spot picking.....	59
2.14. In-Gel trypsin digestion protocol	59
2.15. Mass spectrometry.....	60
2.16. Experimental design for samples used for 2D-gel electrophoresis	62
2.17. Estimation of leaf area and plant biomass.....	65

2.17.1. Leaf area.....	65
2.17.2. Plant biomass	65
2.18. Visualization of leaves using normal and infrared camera	65
3. Results.....	66
3.1. Healthy plants.....	67
3.1.1. Protein extraction from pea roots.....	67
3.1.1.1. 1D-gel electrophoresis (SDS PAGE).....	67
3.1.1.2. Purification of protein extracts.....	70
3.1.1.3. Conductivity of extracted protein solutions	71
3.1.1.4. Protein quantification.....	73
3.1.1.5. 2D-gel electrophoresis	74
3.1.2. Protein extraction from pea leaves.....	75
3.1.2.1. Protein purification.....	75
3.1.2.2. Protein quantification.....	75
3.1.2.3. 1D-gel electrophoresis (SDS PAGE).....	76
3.1.2.4. 2D-gel electrophoresis	77
3.1.3. Effect of plant age on the amount of protein in pea leaves.....	79
3.2. Infected plants	81
3.2.1. Downy mildew.....	81
3.2.1.1. Pea inoculation.....	81
3.2.1.2. Protein extraction	82
3.2.1.3. 1D-gel electrophoresis (SDS PAGE).....	83
3.2.1.4. 2D-gel electrophoresis	85

3.2.1.5. Mass spectrometry and data analysis	90
3.2.2. <i>Fusarium</i> root rot	94
3.2.2.1. Pea inoculation	94
3.2.2.2. Protein extraction and purification.....	96
3.2.2.3. Protein quantification	97
3.2.2.4. 1D-gel electrophoresis (SDS PAGE).....	98
3.2.2.5. 2D-gel electrophoresis	99
3.2.2.6. Mass spectrometry and data analysis	104
3.2.3. Root knot nematode (<i>Meloidogyne hapla</i>).....	108
3.2.3.1. Pea inoculation	108
3.2.3.2. Protein extraction, quantification and purification.....	110
3.2.3.3. 1D-gel electrophoresis (SDS PAGE).....	113
3.2.3.4. 2D-gel electrophoresis	114
3.2.3.5. Mass spectrometry and data analysis	119
3.3. Comparison between proteins extracted from roots of healthy and <i>M. hapla</i> , <i>F. solani</i> and <i>P. viciae</i> , infected plants	125
3.3.1. 1D-gel electrophoresis (SDS PAGE).....	126
3.4. Summary of protein identification: protein biomarkers for specific diseases	127
3.5. Effect of <i>M. hapla</i> and <i>F. solani</i> on pea growth.....	132
3.5.1. Leaf area and appearance	132
3.5.2. Plant biomass	134
3.5.2.1. Fresh and dry weight of shoot system.....	134
3.5.2.2. Fresh and dry weight of root system	135

3.6. Diagnosis of root knot and root rot diseases of peas by processing of leaf images.....	137
3.6.1. Imaging leaves with a digital camera.....	137
3.6.2. Imaging leaves with infra-red camera.....	139
4. Discussion	140
4.1. Inoculation of plants and maintenance of the pathogens	141
4.1.1. Inoculation with <i>Peronospora viciae</i> f. sp. <i>pisi</i>	141
4.1.2. Inoculation with <i>Fusarium solani</i> f. sp. <i>pisi</i>	141
4.1.3. Inoculation with <i>Meloidogyne hapla</i>	142
4.2. Effect of root infection on plant growth	143
4.3. Effect of age on the amount of protein in pea leaves	147
4.4. Protein extraction, purification and quantification.....	148
4.4.1. Protein extraction	148
4.4.1.1. Protein extraction from pea roots.....	149
4.4.1.2. Protein extraction from pea leaves.....	152
4.4.2. Protein purification.....	152
4.4.3. Protein quantification	154
4.5. Comparison of proteins extracted from healthy and infected pea plants	155
4.5.1. Protein extracted from roots of pea plants infected by <i>P. viciae</i>	155
4.5.2. Protein extracted from roots of pea plants infected by <i>F. solani</i>	156
4.5.3. Protein extracted from roots of pea plants infected by <i>M. hapla</i>	156

4.6. Separation of proteins by electrophoresis.....	157
4.6.1. Separation of proteins extracted from roots of pea plants infected by <i>P. viciae</i> .	158
4.6.2. Separation of protein extracted from leaves of pea plants infected by <i>F. solani</i> .	159
4.6.3. Separation of protein extracted from leaves of pea plants infected by <i>M. hapla</i> .	161
4.7. Identification of proteins	161
4.7.1. Identification of proteins extracted from pea roots	162
4.7.2. Identification of proteins extracted from pea leaves	165
4.7.2.1. Identification of proteins extracted from leaves of <i>F. solani</i> infected pea plants.....	165
4.7.2.2. Identification of proteins extracted from leaves of <i>M. hapla</i> infected pea plants.....	167
4.8. Role of proteins with altered abundance	168
4.9. Detection of infection and identification of the cause.....	179
4.9.1. Specific protein biomarkers from roots of <i>P. viciae</i> infected plants.....	179
4.9.2. Specific protein biomarkers from leaves of <i>F. solani</i> infected plants	180
4.9.3. Specific protein biomarkers from leaves of <i>M. hapla</i> infected plants	180
4.10. Digital imaging of leaves for diagnosis of root diseases.....	181
5. Conclusion	184
6. Future work	186
7. References	190

List of figures

- Figure 1.1. Image of field of peas infected by *P. viciae* (a), with bare patches of soil where infected plants have been killed, with close-up view of single infected pea plant in (b). 8
- Figure 1.2. Image of field of peas infected by *Fusarium* root rot (a), with close up of single infected plants (b). 13
- Figure 1.3. Images of field of peas infected by *Meloidogyne hapla* (a), with close-up view of roots of a single infected plant (b). 15
- Figure 1.4. Techniques available for plant disease diagnosis. 21
- Figure 1.5. Diagram showing the method of using a Pocket Diagnostic kit. 23
- Figure 1.6. The four levels of protein structure. 26
- Figure 2.1. Mini gel system (Atto Corporation, Japan)
(a) Electrophoresis power supply, (b) Electrophoresis chamber. 51
- Figure 2.2. (a) Six strip holders, (b) Ettan IPG-Phor system (GE Healthcare). 53
- Figure 2.3. Ettan Dalt Six Gel Caster (a) Screw, (b) triangular rubber, (c) spring clamp, (d) gasket, (e) separator sheet. 55

Figure 2.4. (a) Electrophoresis Power Supply EPS 601, (b) 2D-gel tank for SDS-PAGE separation.	58
Figure 2.5. Flow chart summarising the inoculation and protein extraction protocol used to prepare samples for 1D- and 2D-gel electrophoresis.	63
Figure 2.6. Experimental design for 2D gels of proteins from pea plants inoculated with different pathogens. H = gels of healthy and I = gels of infected (<i>P. viciae</i> , <i>F. solani</i> or <i>M. hapla</i>) pea plants.	64
Figure 3.1. 1D gel of proteins extracted from pea roots by six methods: 1, TCA-acetone; 2, SDS; 3, SDS-acetone; 4, Amey; 5, BPP; 6, Brigham.	68
Figure 3.2. A representative 1D gel shows 4 different amounts of proteins (5, 10, 15, 20 μ l), extracted using the TCA-acetone method. MW = molecular weight markers, KDa.	69
Figure 3.3. 1D gel of proteins extracted from pea roots using the Amey method in 3 biological replicates (1, 2, 3) and either treated using the GE Healthcare 2D Clean-up Kit (clean protein) or untreated (non-clean protein).	70
Figure 3.4. 1D gel of proteins extracted from pea roots using the Amey method in 3 biological replicates (1, 2, 3) and either treated using the Zeba De-salt Spin Columns (Z) or untreated (N).	71

Figure 3.5. 2D gel of proteins extracted from roots of healthy pea plants using the Amey method.	74
Figure 3.6. 1D gel of protein extracts from pea leaves of healthy plants using the Amey (A) and Giavalisco (G) methods, from 3 biological replicate samples.	77
Figure 3.7. 2D gels of proteins extracted from leaves of healthy pea plants using the (a) Amey method and (b) Giavalisco method.	78
Figure 3.8. Gels of 3 biological replicates of protein extracts from leaves of healthy pea plants at four (1a, b, and c) and six weeks (2a, b, and c) after sowing seeds.	80
Figure 3.9. Sporulating <i>Peronospora viciae</i> infection of pea petiole (arrow) and stipule (*).	81
Figure 3.10. <i>P. viciae</i> infected stipule (arrow) and leaf (*) of pea.	82
Figure 3.11. Comparison of root protein concentration in extracts from roots of healthy plants (H) and plants infected by <i>P. viciae</i> (I), extracted using the TCA-acetone (Natarajan <i>et al.</i> , 2005) and the Amey methods (Chuisseu <i>et al.</i> , 2007).	83
Figure 3.12. 1D gel comparing proteins extracted from roots of <i>P. viciae</i> infected (I) and non-infected (N) pea plants using the TCA-acetone method.	84

Figure 3.13. 1D gel comparing proteins extracted from roots of *P. viciae* infected (I) and non-infected (N) pea plants using the Amey method. 85

Figure 3.14. 2D gels of 3 biological replicates of proteins extracted from roots of healthy (1a, b, c) and *P. viciae* infected (2 a, b, c) pea plants. 87

Figure 3.15. Images of 2D gels of proteins extracted from pea roots from (a) healthy plants and (b) plants infected by *P. viciae* (gels 1c and 2c from Figure 3.14) showing in (a) nine protein spots selected for identification using mass spectrometry. Some samples of identified spots are shown as enlarged images. Spot number is the same as in Fig. 3.16 and Table 3.5. PI= pH gradient from 3-10; MW = molecular weight (KDa). 88

Figure 3.16. Images of three protein spots present in 2D gels of roots of healthy pea plants (square box, numbered as in Fig. 3.15a) and equivalent position on 2D gel of proteins from roots of *P. viciae* infected plants (circles), detected using PDQuest software. For the proteins from roots from healthy plants, the 3D view of the spots and the relative abundance of the protein in each of 3 biological replicates is as shown by PDQuest software. 89

Figure 3.17. *Fusarium solani* f. sp. *pisi* micro- (arrowheads) and macro-conidia (arrows). 94

Figure 3.18. Three healthy pea plants on right and three plants infected by *F.*

<i>solani</i> on left, four weeks after inoculation by the soil inoculation method, showing stunting of shoots and roots following infection.	95
Figure 3.19. Healthy pea plant on right and plant infected by <i>F. solani</i> on left, four weeks after inoculation by the seed inoculation method.	96
Figure 3.20. A representative 1D gel comparing protein extracted from leaves of healthy (H) and <i>F. solani</i> infected (I) pea plants using the Giavalisco method.	98
Figure 3.21. Gels of 3 biological replicates of proteins extracted from leaves of healthy (1 a, b, c) and <i>F. solani</i> infected (2 a, b, c) pea plants.	101
Figure 3.22. Images of 2D gels of protein extracted from leaves from healthy (a) and <i>F. solani</i> infected (a) pea plants (gels 1 and 2a from Figure 3.21) showing 15 spots selected for identification using mass spectrometry. Some samples of identified spots are shown as enlarged images. Spot number is the same as in Fig. 3.23. PI= pH gradient from 3-10; MW= molecular weight (KDa).	102
Figure 3.23. Representation of three protein spots only present in 2D gels of leaves of healthy plants (square box) compared to leaves from <i>F. solani</i> infected plants (circles) detected using PDQuest software, with spot numbers relating to spots in Fig. 3.22. The 3D view of spots and the relative abundance of the protein in each of 3 biological replicates from leaves of healthy plants is as shown by PDQuest software.	103

Figure 3.24. Roots of plant of *Lycopersicon esculentum* cv. Money Maker with galls due to infection by *Meloidogyne hapla*. 108

Figure 3.25. Roots of *P. sativum* plants with (a) discrete *Rhizobium* nodules (arrowed) on healthy plants and (b) galls due to infection by *M. hapla* causing thickening of the roots (arrowed). 109

Figure 3.26. Comparison between proteins extracted from leaves of healthy (H) and *M. hapla* infected pea plants (I) using the Giavalisco method, three weeks post-inoculation. 114

Figure 3.27. Gels of 3 biological replicates of protein extracts from leaves of healthy (1a, b, and c) and *M. hapla* infected (2a, b, and c) pea plants, six weeks after inoculation. Selected proteins identified by PDQuest analysis to be present constantly in extracts from either healthy or infected are indicated in green and red respectively. An example of one protein that is present in all gels is indicated by a yellow circle. 116

Figure 3.28. Images of 2D gels of proteins extracted from leaves of healthy (a) and *M. hapla* infected (b) pea plants (gels 1c and 2c from figure 3.27) showing 20 protein spots selected for identification using Q-TOF mass spectrometry. Some samples of identified spots are shown as enlarged images. Spot number is the same as in Fig.3.29 and Table 3.11. PI= pH gradient from 3-10; MW = molecular weight (KDa). 117

Figure 3.29. Representation of three protein spots only present in 2D gels of proteins from leaves of healthy plants (square box) compared to leaves of *M. hapla* infected plants (circles) detected using PDQuest software. 3D view of spots and the relative abundance of the protein in each of 3 biological replicates from leaves of healthy plants is as shown by PDQuest software. 118

Figure 3.30. Comparison of protein concentrations in extracts from roots of healthy pea plants and plants four weeks after infected by *M. hapla*, *F. solani* and *P. viciae*, using the Amey method (Chuisseu *et al.*, 2007). 126

Figure 3.31. 1D gel of proteins extracted from roots of healthy pea plants and plants four weeks after infected by different pathogens (15 µl loaded in each lane). MW, molecular weight; H, roots from healthy plants; D, plants infected by *P. viciae*; F, plants infected by *F. solani*; N, plants infected by *M. hapla*. 127

Figure 3.32. Comparison of leaf area of four week-old healthy pea plants and plants infected by *F. solani* and *M. hapla* (n = 3). 132

Figure 3.33. Comparison of leaf and stipule size and number from three replicate four week-old pea plants, that are healthy (1) and infected by *M. hapla* (2) and *F. solani* (3). 133

Figure 3.34. Comparison of shoot fresh weights of healthy and *F. solani* and *M. hapla* infected pea plants four weeks after sowing seeds. 134

Figure 3.35. Comparison of shoot dry weights of healthy and <i>F. solani</i> and <i>M. hapla</i> infected pea plants four weeks after sowing seeds.	135
Figure 3.36. Comparison of root fresh weights of healthy and <i>F. solani</i> and <i>M. hapla</i> infected pea plants, four weeks after sowing seeds.	136
Figure 3.37. Comparison of root dry weights of healthy and <i>F. solani</i> and <i>M. hapla</i> infected pea plants, four weeks after sowing seeds.	136
Figure 3.38a. Leaves from healthy (h) and <i>F. solani</i> (f) and <i>M. hapla</i> (m) infected pea plants imaged with a normal digital camera.	138
Figure 3.38b. Leaves from healthy and <i>F. solani</i> and <i>M. hapla</i> infected pea plants imaged as in Figure 3.38a but with the image viewed using the red channel set at 21%, and superimposed on the original image.	138
Figure 3.38c. Leaves from healthy and <i>F. solani</i> and <i>M. hapla</i> infected pea plants imaged as in Figure 3.38b but with the red channel set at 44%.	139

List of Tables

Table 2.1. Cultivars of pea (<i>Pisum sativum</i> L.) used for inoculation with <i>Peronospora viciae</i> , <i>Meloidogyne hapla</i> and <i>Fusarium solani</i> .	39
Table 2.2. Cultivars of tomato (<i>Solanum lycopersicum</i> L.) used for maintaining <i>Meloidogyne hapla</i> .	40
Table 3.1. Conductivity of protein solutions extracted from pea roots using the Amey method (Chuisseu <i>et al.</i> , 2007), compared to conductivity of deionised water and NaCl solutions in three replicates of each.	72
Table 3.2. Concentration of protein extracted from roots of healthy pea plants using the Amey and TCA-acetone methods. Data from 3 experiments, each of 3 biological replicates.	73
Table 3.3. Concentration of protein extracted from leaves of healthy pea plants using the Amey and the Giavalisco methods. Data from 3 experiments, each of 3 biological replicates.	76
Table 3.4. Concentration of proteins extracted from leaves of healthy pea plants at 4 and 6 weeks old using the Giavalisco method. Data from 3 experiments, each with 3 biological replicates.	79

Table 3.5. Proteins that differed in abundance in pea roots in response to infection of leaves by <i>P. viciae</i> , identified using MALDI-TOF (spot no. 1 and 3) and ESI Q-TOF MS/MS (all other spots). Spots numbered 1-9 are from healthy plants, as labelled in gels in Fig. 3.15a, and decreased in abundance by at least two fold following infection.	92
Table 3.6. Concentration of protein extracted from leaves of <i>F. solani</i> infected and healthy pea plants using the Giavalisco method. Data from 3 experiments, each of 3 biological replicates.	97
Table 3.7. Proteins that differed in abundance in pea leaves in response to infection of roots by <i>F. solani</i> , identified using ESI Q-TOF MS/MS. Spots numbered 1-11 are from healthy and protein 12 from infected plants, as labelled in gels in Fig. 3.22; proteins 1-11 were decreased in abundance by at least four fold, while protein 12 was increased by at least four fold following infection.	106
Table 3.8. Concentration of protein extracted from leaves of <i>M. hapla</i> infected pea plants three weeks after inoculation and leaves of healthy pea plants using the Giavalisco method. Data from 3 experiments, each of 3 biological replicates.	111
Table 3.9. Concentration of protein extracted from leaves of <i>M. hapla</i> infected pea plants six weeks after inoculation and leaves of healthy pea plants using the Giavalisco method. Data from 3 experiments, each with 3 biological replicates.	112

Table 3.10. Concentration of protein extracted from leaves of four weeks old healthy pea plants using the Giavalisco method, in crude extracts and after cleaning with the 2D Clean-up Kit. Data from 1 experiment, with 3 biological replicates.	113
Table 3.11. Proteins that differed in abundance in pea leaves in response to infection of roots by <i>M. hapla</i> , identified using ESI Q-TOF MS/MS. Spots numbered 1-17 from healthy and 18-20 from infected plants, as labelled in gels in Fig. 3.28; proteins 1-17 were decreased in abundance by at least four fold, while proteins from 18-20 were increased by at least four fold following infection.	121
Table 3.12. Summary of proteins identified in pea roots and leaves as a result of infection by <i>P. viciae</i> , <i>F. solani</i> and <i>M. hapla</i> .	129

1. Introduction

1.1. Background

The pea plant (*Pisum sativum*) is recognized as one of the earliest cultivated species, with archaeological evidence indicating that it was first grown in near eastern and Greek Neolithic settlements nearly 6000 BC (Marx, 1977; Kraft *et al.*, 1998). Some wild and primitive cultivated forms of peas were also found in central Asia, around the Mediterranean Sea and in Ethiopia (Hedrick *et al.*, 1928). The migration of humans is the main factor for the dispersal of peas throughout the world. Pea seeds were simple for early humans to collect and dry, and were a rich source of protein and carbohydrates (Hedrick *et al.*, 1928). Peas became a very important crop in the North of Europe especially in the middle ages where they were grown as an edible grain, and in England became a chief crop (Hedrick *et al.*, 1928). Significant improvements to yield were made in England during the nineteenth century that resulted in several improved cultivars that remain commercially important today (Hedrick *et al.*, 1928; Kraft *et al.*, 1998).

Peas for human consumption today are divided into two main categories; those harvested as pods for eating fresh, and those harvested as seeds from vining peas for processing in cans or for freezing. Dried peas are also harvested when fully mature and used for human consumption. An increasing amount of peas are grown for animal consumption (Cousin, 1997; Kraft *et al.*, 1998).

Pea is one of the important world trade crops and represents about 40% of the total world trade in legumes (Oram and Agcaoili, 1988). Its production has been increased in some developed countries (Grunwald *et al.*, 2004), and it has become the fourth most important legume after soybean, groundnut and *Vicia faba* beans (Hulse, 1994). Pea is considered to be an important source of protein with 21%-25% (Schatz and Endres, 2009) of the dry weight stated as the protein content, and

it also contains a large amount of carbohydrates at about 56.6% of dry seed weight (Bressani and Elias, 1988). It is also a rich source of amino acids such as lysine and tryptophan (Schatz and Endres, 2009).

Together with the high levels of carbohydrates and low percentage of fibers, 86-87% of peas are digestible nutrients, which make it an excellent livestock feed (Schatz and Endres, 2009). Also it contains less trypsin inhibitors compared to soybean which means it can be fed directly to livestock without having to go through the extrusion heating process (Schatz and Endres, 2009). The total production of dry peas worldwide reached around 8.127 million metric tonnes between a 1979-1981, and increased to 14.529 in 1994, while the area on which pea was grown increased from 7.488 to 8.060 million hectares for the same years (Anon., 1994). Peas typically tolerate cold temperature down to -2°C in the seedling stage, with some winter hardy varieties tolerating down to -10°C (Slinkard *et al.*, 1994). The optimum temperature for peas in the vegetative and reproductive stages is from 16°C to 21°C during the day and 10° to 16°C at night, whilst temperatures above 27°C adversely affect pollination and growth. Pea seeds are typically sown in the spring in temperate climates, but can be grown in the middle of summer where relatively low temperatures and a good rainfall are available or the crop is irrigated. Sandy loam soil is preferred for very early crops; otherwise a well-drained clay loam or slit loam is preferred to ensure a large yield where earliness is not a factor (Duke, 1981). The growing duration from sowing to harvest depends on the climate. For example, in semi-arid regions it is from 80 to 100 days, while in humid and temperate areas it is up to 150 days (Davies *et al.*, 1985).

Peas form a symbiosis with *Rhizobium* resulting in root nodules where nitrogen is fixed. This reduces the nitrogen fertilizer requirements of subsequent crops. For

example, the N requirements for maize is reduced by 20-32 kg/ha in India, compared to when maize followed wheat or a fallow year, respectively (Davies *et al.*, 1985). In France, peas returned about 50 kg/ha of N to the soil (Davies *et al.*, 1985). Pea crops in the USA fixed from 71 kg/ha in Alabama to 119 kg/ha in Wisconsin (Mahler *et al.*, 1988).

In Europe, the important production areas for peas are France, Russia, Ukraine, Denmark and the UK. Other parts of the world where peas are an important crop include China, India, Canada, USA, Chile, Ethiopia and Australia (Anon., 1994). In addition to that pea is one of the common vegetables grown in Libya for human consumption as dry or fresh seeds (Al-Masri, 2000).

1.2. Pea diseases

Peas are infected by several bacteria, fungi, oomycetes, viruses and nematodes, which can significantly decrease crop yield and quality. Some of these pathogens cause soil-borne diseases, which include infections of roots and stems of seedlings and mature plants, as well as systemic diseases of the haulm that can develop from root infections (Engqvist, 2001; Grunwald *et al.*, 2004). Seedling diseases are caused by *Pythium* spp. and *Rhizoctonia solani* Kuhn. Root rot is caused by *Aphanomyces euteiches* Drechs, *Fusarium solani* f. sp. *pisi* (Jones) Snyder & Hans, and *Thielaviopsis basicola* (Berk & Broome) Ferrans. Vascular wilt diseases are caused by *Fusarium oxysporum* f. sp. *pisi* (Van Hall) Snyder & Hans. The majority of nematode diseases are caused by *Heterodera goettingiana* (Liebscher), *Meloidogyne* spp. and *Pratylenchus penetrans* (Cob) Filip & Schunr. Stek (Grunwald *et al.*, 2004).

The management of soil-borne diseases is difficult because these pathogens are soil inhabitants (Grunwald *et al.*, 2004). Measures to control these diseases include growing resistant cultivars, using disease-free seeds and seed treatment with pesticides, crop rotation, suppression, solarisation and increasing the soil organic matter (Grunwald *et al.*, 2004).

Foliar pathogens include *Sclerotinia sclerotiorum* (Lib) De Bary which causes white mould, *Erysiphe pisi* D C which causes powdery mildew, *Peronospora viciae* (Berk) Casp causing downy mildew, *Botrytis cinerea* Pers Fr. causing grey mould, and *Uromyces faba* (Grev) Fuckel causing pea rust. Big losses throughout the world are caused by downy mildew diseases (Clark and Spencer-Phillips, 2000; Dang and Panwar, 2004). In addition, *Ascochyta* blight is caused by a complex of three fungi, *Ascochyta pisi* Lib., *Mycosphaerella pinodes* (Brek & Blotam) Vesterger and *Phoma medicaginis* var *pinodella* (Jones) Boerema. Bacterial diseases of pea leaves are caused by *Pseudomonas syringae* pv. *pisii* (Sackett) Young, Dye and Wilkie, and *Pseudomonas syringae* pv. *syringae* (Grunwald *et al.*, 2004). Management of these diseases is different than for the soil borne diseases. Resistance is available as a strategy to control many foliar diseases such as the bacterial pathogens and fungal diseases such as powdery mildew and *Ascochyta* blight. In addition to this strategy of management, fungicides and bactericides also are effective with these diseases (Grunwald *et al.*, 2004). Previously, it was thought that there were more than 50 virus diseases affecting the pea crop (Grunwald *et al.*, 2004). Recent data indicate that some of these diseases are caused by one or more strains of the same virus, so the number of different viruses affecting peas has been reduced to about 25 (Grunwald *et al.*, 2004). Six of these viruses are internationally important, including Alfalfa Mosaic Virus (AMV), Bean Leaf Roll Virus (BLRV),

Pea Enation Mosaic Virus (PEMV), Pea Streak Virus (PeSV), Red Clover Vein Mosaic (RCVMV) and Pea Seed Borne Mosaic Virus (PSbMV).

These viruses each have a different ecology because of different methods of transmission. Some species of weeds and legumes such as alfalfa and clover species become a natural source of inoculums. Pea viruses are transmitted by different vectors including aphids, nematodes, thrips and beetles, whilst others are transmitted by seeds. Viruses produce different symptoms which can vary for the same virus disease, so diagnosis of viral diseases visually is very difficult (Schroeder *et al.*, 1959). A single virus is able to produce different symptoms on different cultivars of the pea crop (Schroeder *et al.*, 1959).

Prevention is the most effective method to control pea viral diseases in the field. These methods include the use of virus free seeds for PSbMV, in addition to avoiding planting pea crops near alfalfa or clover fields that can act as reservoirs of pea viruses. Aphid control is another important measure to reduce the spread of virus. Resistant and tolerant cultivars can also be planted (Grunwald *et al.*, 2004).

1.2.1. Pea downy mildew

Pea downy mildew is caused by the Oomycete *Peronospora viciae* (Berk) de Bary which belongs to the family Peronosporaceae in the class Oomycetes. The oomycetes are placed in the kingdom Straminipila, and are closely related to golden-brown algae (Dick, 2002). Other plant pathogenic oomycetes include species within the genera *Phytophthora*, *Pythium* and *Aphanomyces* (Alexopoulos *et al.*, 1996).

Oomycetes are characterized by producing conidiophores, if bearing asexual spores called conidia, and sporangiophores if bearing sporangia germinating to form

zoospores (Dick, 2002). These sporophores are dichotomously branched at acute angles and taper to curved pointed tips where the spores are produced. In *P. viciae*, sexual oospores are produced within senescent infected host tissue. Oospores are spherical in shape, light brown to yellowish-pink in colour and 25-37 µm in diameter (Kraft *et al.*, 1998).

The genus *Peronospora* produces conidiospores and conidia, which germinate directly to form germ tubes. Downy mildew pathogens such as *P. viciae* prefer cool moist conditions that are usually present in the early part of the season (Matthews, 1981), and this pathogen has several hosts including peas, broad beans, alfalfa and vetch (Farr *et al.*, 1989). The oospores of downy mildew can survive in the soil for 10-15 years, and they are the primary source of systemic and local infections at the start of the growing season. During periods of high humidity, infection of pods by conidia can occur without foliage symptoms. The infected pods are deformed and a mass of mycelia can be present on the pod wall, with oospores forming in this mycelial growth. This pathogen however is not transmitted via seeds (Stegmark, 1990).

Pea downy mildew is a common disease in the North Europe where weather is suitable (Fig. 1.1a, b). It has been recorded as a particular problem in the UK, Sweden, Norway, Australia and New Zealand (Dixon, 1981).



Figure 1.1. Image of field of peas infected by *P. viciae* (a), with bare patches of soil where infected plants have been killed, with close-up view of single infected pea plant in (b). Source of image: Downy Mildew Research Group, UWE.

This disease also occurs in the USA especially in the early part of the growing season, but it is not economically important (Reiling, 1984). It has also been

recorded as a problem in Australia and New Zealand (Dixon, 1981; Davidson and Ramsey, 2000). Smith (1884; reported in Campbell, 1935) first reported *P. viciae* growing in pods and seeds, Linford (1929; reported in Campbell, 1935) first reported the oospore stage in leaves, stems and pods, whilst Melhus (1931) found the mycelium in the seed coats of infected pods. In severe infections the plant usually is killed before flowering (Matthews, 1981), while late infection may only affect the apical plant parts. *P. viciae* causes several types of symptoms on infected pea plants. On the surface of infected leaves greenish-yellow to brown patches appear as angular areas delimited by veins. Grey conidia are borne on conidiophores on the lower surface of leaves opposite to the lesion on the upper surface, giving the characteristic downy symptoms. Seedlings can become infected systemically by oospores in the soil. This results in conidial sporulation that covers a major part of the plant surface. Local infection is recognized as sporulation on the leaves, tendrils or stems and develops from conidia that land on the plant surface. In high humidity periods pods can be infected. The infected pods are deformed as result of infection and covered with yellow to brownish areas (Matthews, 1981; Kraft and Kaiser, 1993; Fallon and Sutherland, 1996). Pod infection is recognized as yellow lesions on the pod surface, and also epithelial proliferation on the endocarp. Also pod infection causes abortion of seeds and brown-discoloured small peas with a bitter taste (Stegmark, 1994). This type of infection develops from conidia deposited on young pods rather than by mycelial growth through the peduncle and pedicel (Mence and Pegg, 1971).

Most pea cultivars are not resistant to downy mildew disease, with yield loss of up to 55% being reported from this disease alone (Clark and Spencer-Phillips, 2000). But some such as Dark Skin Perfection are more resistant than others. Even this

cultivar, however, can be affected severely when conditions are optimum for downy mildew (Olofsson, 1966; Stegmark, 1988).

To control downy mildew several methods should be used. In addition to resistance, crop rotation with long periods between pea crops can be one of methods to control this disease. Fungicides specific for oomycetes can be applied to control pea downy mildew, but the pathogen has become resistant to systemic fungicides such as Metalaxyl and derivatives such as Metalaxyl-M. These fungicides have been used as an effective seed treatment to reduce the primary systemic infection (Stegmark, 1990), and can be used in rotation with other protectant fungicides such as chlorothalonil (Grunwald *et al.*, 2004). Another way to control pea downy mildew is by removing the crop debris containing oospores or by deep ploughing, so the oospores are buried below germinating seeds to avoid early systemic infection (Kraft *et al.*, 1998).

1.2.2. *Fusarium* root rot

Fusarium root rot of pea is caused by the fungus *Fusarium solani* f. sp. *pisii*. It was first reported as a severe disease in Minnesota (Bisby, 1918) and Wisconsin (Jones, 1923) in the USA and at the same time in Europe (Butler and Jones, 1949; Buxton, 1955). *F. solani* is now regarded as an important pathogen that effects pea production in the UK, Denmark, France (Biddle, 1984; Oyarzun *et al.*, 1993; Persson *et al.*, 1997). Also it is an important disease in the Pacific Northwest in dry and irrigated lands (Kraft *et al.*, 1981). There are no accurate data on crop losses due to this pathogen, but Kraft and Berry (1972) estimated that yields were reduced by 30% in pea field plots infected artificially, compared with non-infected plots in the same field. Basu *et al.* (1976) reported that losses in processing peas in five

Canadian provinces were from 35% to 57% in experimental plots, with losses of peas in the USA estimated at 10% to 50% (Kraft and Kaiser, 1993). Symptoms in the field are patches of dead and chlorotic plants (Fig. 1.2), which are very similar to infection by *P. viciae* (Fig. 1.1) and *M. hapla* (Fig. 1.3). This disease affects several hosts by causing different diseases such as root rot of pea, branch blight of mulberry trees, and root rot of ginseng (Matuo and Snyder, 1972). *Nectria haematococca* Berk and Broome is the perfect stage of the pathogen, which has been reported in Japan only (Matuo and Snyder, 1972) where it is the casual pathogen of branch blight of mulberry trees (*Morus* spp.). *Fusarium* root rot of pea is distinct from *Fusarium* wilt, caused by *Fusarium oxysporum*, but usually occurs in conjunction with other pea diseases (Walker, 1952; Schroeder, 1953; Zaumeyer, 1962; Kraft and Roberts, 1969). In pea seedlings, the initial point of infection is the cotyledonary attachment area, the below-ground epicotyls, and the upper taproot (Allmaras *et al.*, 1988). Later the infection extends up and down the root, and the severity of root damage depends on soil conditions (Kraft *et al.*, 1981). This disease is enhanced by soil compaction and temperatures above 30°C, acidic soil with pH less than 5.1, moisture contents of soil, in addition to poor soil fertility (Kraft *et al.*, 1981; Allmaras *et al.*, 1988; Kraft *et al.*, 1988). There is some evidence indicating that the soil type and moisture affect the severity of infection (Kraft *et al.*, 1981).

The optimum temperature for *in vitro* growth of *F. solani* is 30°C and the optimum for infection 25-30°C, whilst disease development requires 18°C and above (Walker, 1952; Kraft and Roberts, 1969).

The severity of disease varies due to the pathogen strain (Salt and Delaney, 1985), and it shows symptoms on diseased plants that vary from reddish-brown to black necrosis (Fig. 1.2 b). The vascular system of infected pea roots may show a red

discoloration, but this does not continue above the soil line (Kraft *et al.*, 1998). Symptoms on the shoot system include yellowing and stunted growth (Kraft *et al.*, 1998). Sporodochia bearing conidia on lesions at the base of infected plants are blue-green to buff in colour (Jones, 1923; Walker, 1952). The sporodochia produce three types of spores. Macro-conidia are usually 3 septate, 4.4 to 5 μm by 27 to 40 μm in size, curved and hyaline. Microconidia are one celled, small and elliptical, and are less abundant where borne on sporodochia, but are numerous when the fungus is grown in liquid culture (Walker, 1952; Kraft and Roberts, 1969).

Chlamydospores are produced within hyphae or conidia. They are of different shapes depending on their place of production including intercalary, terminal and single or cantenulate (Walker, 1952; Kraft and Roberts, 1969). No commercial cultivars are resistant to *Fusarium* root rot (Grunwald *et al.*, 2003), where, for example, chemical seed treatment and plant resistance achieve partial control (Grunwald *et al.*, 2004). There are some attempts of using biological control with fungal antagonistic or bacteria, but none of them are being used in practice (Kraft *et al.*, 1988).

Methods to control *Fusarium* root rot include a good tillage procedure to prevent the compaction of soil (Grunwald *et al.*, 2003), increasing the soil moisture and using high quality seeds (Matthews and Whitbread, 1968; Short and Lacy, 1976), crop rotation with 5 year intervals (Reinking, 1942; Schroeder, 1953), and increasing soil fertility to reduce losses in heavy soil (Reinking, 1942).



Figure 1.2. Image of field of peas infected by *Fusarium* root rot (a), with close up of single infected plants (b), with the severity of infection increasing from right to left. Source of images, <http://www.google.co.uk/imges?imgurl=http://plant-> (North Dakota (a) and Ohio State University (b), USA).

1.2.3. Nematode diseases

Plant parasitic nematodes mostly parasitise roots, although some are parasitic on aerial plant parts, with a few seed-transmitted. All agricultural plant species are affected by one or more species of nematode (Oka *et al.*, 2000), with root knot nematodes and cyst nematodes of the genera *Heterodera* and *Globodera* being the most important (Sasser, 1980; Oka *et al.*, 2000). The financial loss due to nematode infections is very difficult to determine accurately (Burrows *et al.*, 1998), but have been estimated to be \$157 billion worldwide (Abad *et al.*, 2008).

Recently, nematode diseases of vegetables have become economically very important and sometimes reach catastrophic levels, with complete crop loss (Jensen, 1972). Most plant parasitic nematodes are cosmopolitan in distribution, whilst others are restricted to specific climate zones (Jensen, 1972). Pea is affected by more than 20 different genera of plant parasitic nematodes. These include pea cyst nematode *Heterodera gottingiana*, root knot nematode especially *M. incognita* (Kofoid and White) Chitwood, and root-lesion nematodes of the genus *Pratylenchus* (Johnson and Fassuliotis, 1984; Riggs and Niblack, 1993). Other important species include *M. arenaria*, *M. hapla*, *M. javanica*, *H. schachtii*, *H. trifolii* and *Rotylenchus reniformis* (Gill, 1989).

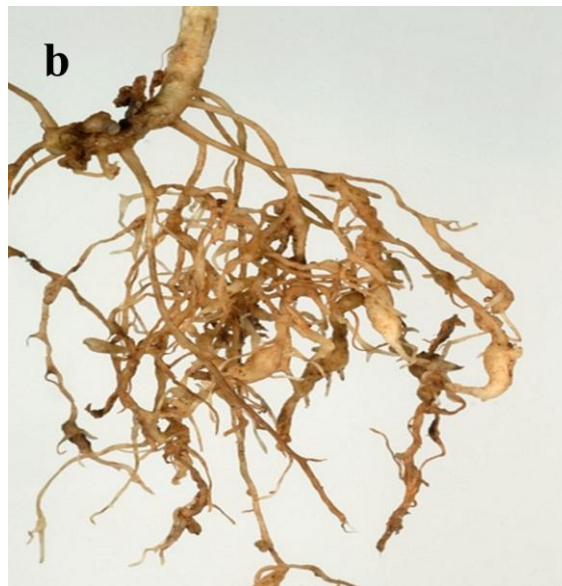


Figure 1.3. Image of field of peas infected by *Meloidogyne hapla* (a), with close-up view of roots of a single infected plant (b). Source of images, <http://www.visualsunlimited.com/image/I0000rVlzqz5PiSwightboxes>

Species of *Meloidogyne* are distributed all over the world and have a wide host range that includes nearly all crop plants (Sasser, 1977; Barker *et al.*, 1985; Sasser and Johnson, 1985; Opperman *et al.*, 2008), and are estimated to cause \$50 billion losses in crop damage every year (Opperman, 2008). About 100 species of *Meloidogyne* spp. have been described (Bridge and Starr, 2007), with the four most important being:

M. incognita (Kofoid and White) Chitwood; *M. javanica* (Treub) Chitwood; *M. arenaria* (Neal) Chitwood; and *M. hapla* Chitwood. They are responsible for 95% of all infestations and cause 5% in crop losses all over the world (Taylor and Sasser, 1978). *M. incognita* causes severe losses in pea of up to 33% (Upadhyay and Dwiveddi, 1987), and up to 90% losses in cowpea (Olowe, 2005). *Meloidogyne* species are usually associated in disease complex-like infections with fungal root rot and wilt diseases as they predispose plants to infection (Johnson and Fassuliotis, 1984). Symptoms on infected plants resemble those of water deficiency with wilting and chlorosis (Oka *et al.*, 2000), so it is difficult to diagnose nematode diseases based on above ground symptoms. The gall symptom of root knot nematodes were first recorded by Berkeley in 1855 (according to Jensen, 1972).

M. hapla is known as Northern Root Knot Nematode and occurs in cooler climates, as well as in tropical and subtropical regions (Opperman, 2008). It has a wide host range with over 550 hosts from different varieties of crops including vegetables and weeds (Widmer *et al.*, 1999). In addition, *M. hapla* is able to survive in freezing temperatures (Jenkins and Taylor, 1967), and therefore occurs frequently in cool climates (Brown, 1955; Ichinohe, 1955; Taylor and Buhner, 1958). It is also recorded in New South Wales in Australia (Blake, 1963) which has a hot climate, as well as on winter crops in Mediterranean countries (Lamberti, 1997). This ability to

tolerate warm climates is significant as climate change in Britain is already effecting crop production through quality and disease distribution (Newton and Gregory, 2007). New pests and pathogens recorded recently in the UK include root-knot nematodes (*Meloidogyne* spp.), the bacterium *Erwinia chrysanthium* and the oomycete *Phytophthora ramorum* (Newton and Gregory, 2007). Thus whilst *M. hapla* previously was not known to be as widespread as other species of root knot nematode, as a result of climate changes it is now found in Uganda and other tropical and subtropical regions (Opperman, 2008). *M. hapla* control using chemicals is limited because of high costs during application, and also the effect of the chemicals on the environment and human health (Noling and Becker, 1994).

The *Meloidogyne* life cycle takes about 25 days or more to complete, depending on the temperature. It is very easy to differentiate morphologically between males and females. The males are worm-like and approximately 1.2 to 1.5 mm long and 30 to 36 μm in diameter, while the females are spherical or pear shaped, and about 0.4 to 1.3 mm long by 0.27 to 0.75 mm wide. Mature females lay about 500 eggs in a gelatinous egg mass. The first-stage juvenile (referred to as the J1 stage) is worm-like and develops inside the egg to give second-stage juveniles (J2), which emerge from the egg to the soil after hatching. The J2 juveniles are the only infective stage, and in the presence of a susceptible host they enter the root and become sedentary and sausage-shaped. These J2 nematodes feed by inserting their stylet into the host cells around their head, also secreting saliva into the cells. The role of the saliva is to stimulate the host cells to become enlarged, and also to dissolve some of cell contents to aid feeding through the stylet. After this stage, the second molt takes place to give the third-stage juvenile (J3). These do not have a stylet, and undergo the third molt to give the fourth-stage juvenile (J4). It is in this stage that males and

females can be distinguished. Next the male J4 stages undergo a final molt and move from the root to the soil as adults that are free-living in the soil. The J4 stage female continues to grow and becomes thicker with the final molt taking place to give the adult females that are sedentary. The mature female can produce eggs with or without males (Agrios, 1997). Warm moist soils are suitable for egg hatching, and whilst some eggs can survive for a long period at least one year and in warm regions up to ten generations can be produced in a single year (Rahman, 2003).

Reducing nematode numbers to levels below the damage threshold is the main goal of nematode control (Anon., 1998). Many strategies are used to control nematodes, such as sanitation by burning diseased plants to prevent nematodes from building-up and spreading, crop rotation, a fallow summer, solarisation and organic amendments. Soil sterilization and fumigation can be also used to reduce the nematode populations. Soil sterilization can be by low temperature steaming or high temperature steam treatment of glasshouse soil. The reason for using these two types of steaming is the high temperature steaming of soil (82°C and above) destroys soil nutrients and breaks down soil structure, as well as killing the soil microorganisms. Since both useful and disease causing organisms are removed from the soil, there is little competition for any parasitic fungi that invade the soil after steaming. Therefore, if the fungi that cause damping-off type diseases are introduced into soil sterilized by high temperature steaming, losses of seedlings will be much greater than in unsterilized soil. To overcome these problems methods of low temperature steaming were developed, where the soil is treated for 30 minutes at 60°C . This will eradicate nematodes and other harmful organisms without killing too many of the beneficial organisms in the soil. Chemical fumigation has been

used previously, but has been reduced due to environmental and health problems to humans and animals (Rahman, 2003).

A central part of this control strategy is to accurately diagnose nematode infection and only apply treatments when they are needed. Diagnostic kits, therefore, have the potential for a significant role in Integrated Pest Management (IPM).

Some nematicides used for fumigation, such as di-bromochloropropane (DBCP) and ethylene dibromide (EDB), have been phased out from the market, whilst methyl bromide has been banned in some countries and withdrawn from most by international agreement (Oka *et al.*, 2000). Due to the difficulty of controlling plant parasitic nematodes, however, methyl bromide is still used legitimately controlling some diseases such as in fruit and nut nurseries in the USA (Zasada *et al.*, 2010). The use of other non-fumigant nematicides based on organophosphate and carbamates could be increased as a result of methyl bromide withdrawal, but these bring new environmental problems. Chemicals that might cause problems in the future include Aldicarb, which is used as a nematicide and insecticide, and has the ability to leach into ground water (Oka *et al.*, 2000). High levels of 1,3-dichloropropane (1,3-D), used as a soil fumigant, has been detected in California air where this nematicide is used too much (Oka *et al.*, 2000). Such chemicals are subject to a United States Environmental Protection Agency (USEPA) special review (Oka *et al.*, 2000). Due to these circumstances, IPM becomes important (Oka *et al.*, 2000).

1.3. Diagnosis of plant diseases

The definition of diagnosis is “the process of determining the cause of a problem” (Pscheidt, 2008). In relation to plant diseases, some pathogens can be diagnosed

easily in the field, such as powdery mildew, downy mildew, rusts, smuts, crown gall, canker, and a few virus diseases that give symptoms such as bunchy top, rosette, witches-broom, phyllody and flower colour-breaking. In many diseases the early stages are inconspicuous until the infection level becomes high. In diseases where symptoms include, for example, chlorosis, mosaic, leaf drooping, dwarfing, stunting, necrosis, root rot, wilt, fruit rot, dieback and leaf blight, significant damage has occurred once symptoms appear. To prevent epidemics, diseases should be diagnosed at the early stages of infection, so different techniques are required, and several scientific methods can be applied to identify plant pathogens (Fig. 1.4). One of these is Koch's postulates (Strange, 2003), which are followed to prove that a detected pathogen is the causal agent of the disease. Methods such as this are based on evaluation of the symptoms and are the best ways to diagnose plant diseases (Link *et al.*, 1999; Schaad *et al.*, 2003), but they take time and require skills in observing symptoms and matching to published descriptions.

Advances in technology have brought new techniques for disease diagnosis. Technology based on nucleic acid hybridization, such as use of molecular beacons, PCR, DNA sequencing, dsRNA analysis, and antibody-based methods such as enzyme-linked immunosorbent assay (ELISA) and Western blots, have the ability to identify pathogen species as well as strains that differ genetically and molecularly (Link *et al.*, 1999; Schaad *et al.*, 2003; Strange, 2003). Other methods for identifying pathogens include substrate metabolism, and fatty acid profiles (Strange, 2003). Many of these methods are fast, precise, easy and cheap, but they require pathogen-specific reagents which may be expensive and complicated to produce. Mass spectrometry (MS) is an alternative method that can differentiate peptides unique to a particular plant pathogen, with no need for pathogen-specific reagents.

MS is relatively expensive and generally not available to farmers. In practice though there is a very little application of MS for detection of plant pathogens and disease diagnosis. MS can be used, however, to identify marker proteins that can then be used in cheap, rapid, field-based diagnostic kits.

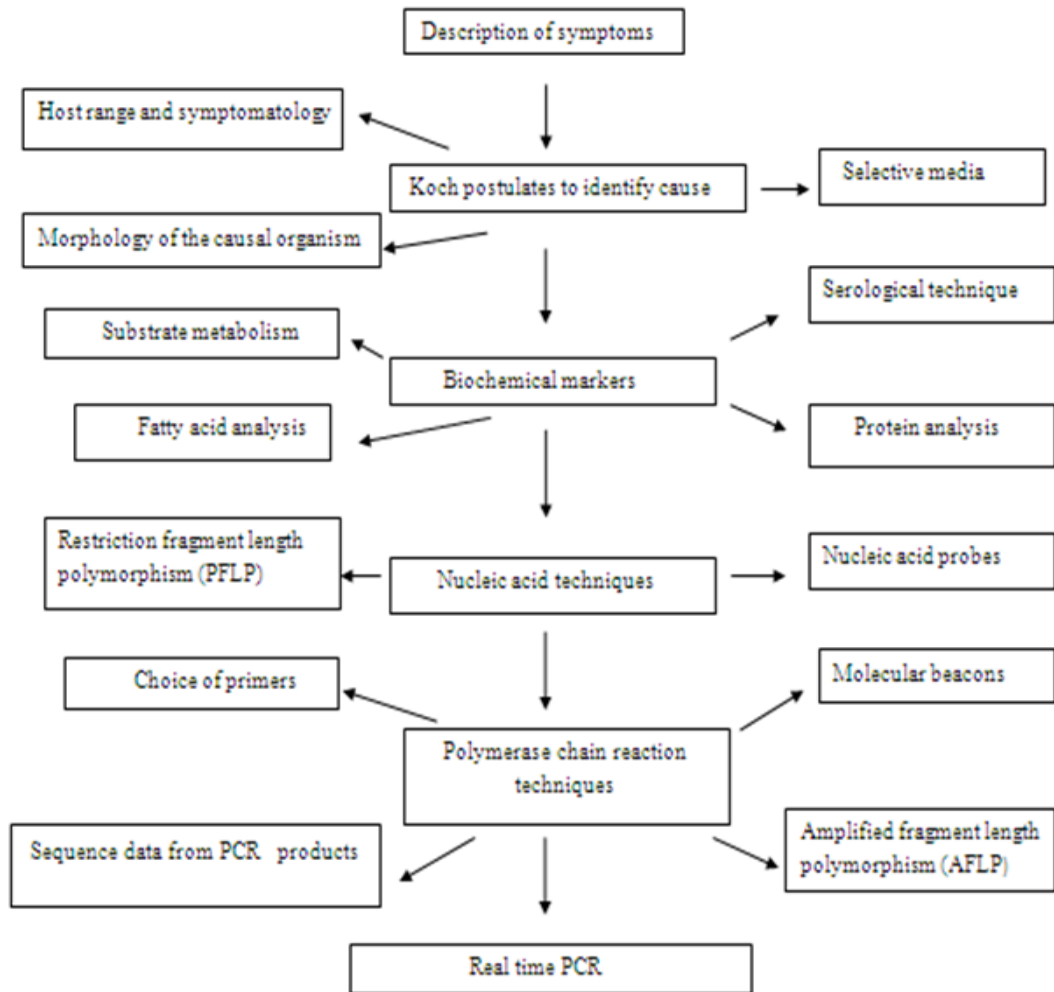


Fig.1.4. Techniques available for plant disease diagnosis (adapted from Strange, 2003).

Recent published guides to disease symptoms include CD-ROMs. For instance, the American Phytopathological Society has a collection of CD-ROMs with digital

images to help in disease diagnosis. Likewise CAB International (<http://pest.cabweb.org>) published a crop protection compendium in 2002, providing information about host range, geographical distribution, biology, ecology and control in addition to images of disease symptoms. These CD-ROMs can be installed in personal portable computers for use in the field (Strange, 2003). Root diseases are more time consuming to diagnose compared to diseases of the shoot system as the roots need to be inspected (Dusunceli and Fox, 1992).

1.4. Plant disease diagnostics for field use

Misdiagnosis of a disease can be very costly to a grower if unnecessary fungicide applications or other control measures are used. It would be beneficial to growers to have rapid and simple disease identification test kits available to make accurate initial disease diagnosis in the field. These test kits can also be helpful in eliminating disease misdiagnosis (Olsen *et al.*, 2011).

Diagnostic kits have been developed to diagnose diseases including viral diseases such as Alfalfa Mosaic Virus, Cucumber Mosaic Virus and Apple Mosaic Virus, bacterial diseases caused by *Erwinia amylovora*, *Xanthomans campestris* pv *pelargoni*, fungal diseases caused by *Pythium* spp., *Rhizoctonia* spp., *Botrytis* spp. and oomycete diseases caused by *Phytophthora* spp. (Anon, 2012b). Each kit is specific to a particular disease. The Pocket Diagnostic kit contains two main parts, which are: the test strip carrying the antibodies and other reaction ingredients securely held in a plastic housing, ensuring it is protected from damage; the extraction bottle and buffer plus ball bearings that are required to break up the plant material, all within a rigid, leak-proof bottle; a plastic pipette for adding 2-3 drops of sample to the test device. This method is easy to use in the field, and gives good

sample extraction across a wide range of plant materials. The Pocket Diagnostic kit adopts similar technology to the highly successful home pregnancy test kit. The tests are specific for individual pathogens and utilise antibodies which have been used in reliable routine laboratory testing for over 20 years. Pocket Diagnostic kit use a unique ball and bottle extraction method which optimises separation of the pathogen from the plant tissues in no more than 60 s (Fig. 1.5).

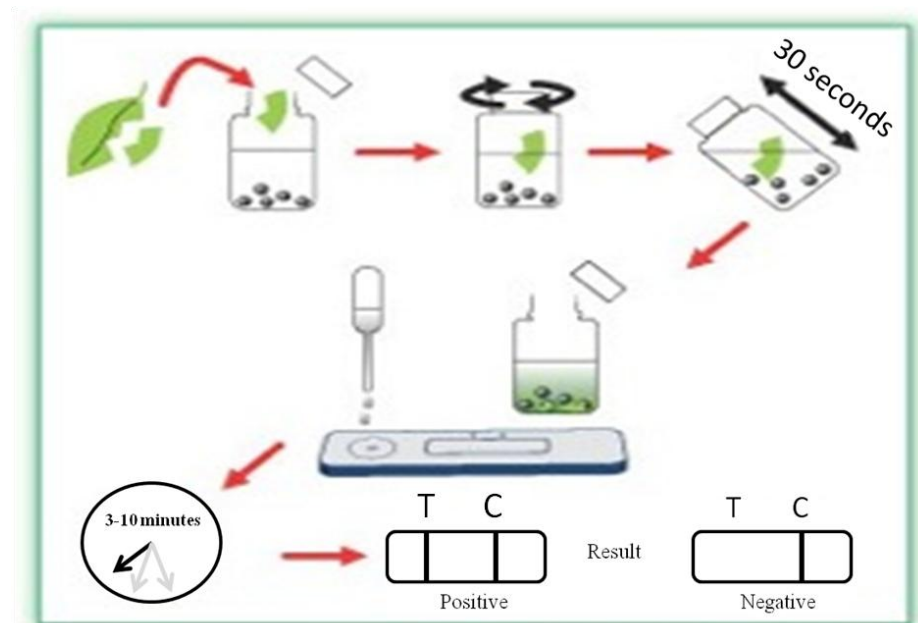


Fig.1.5. Diagram showing the method of using a Pocket Diagnostic kit.

[http://www.pocketdiagnostic.com/uploads/File/Datasheets/Phytophthora Data Sheet Hi%20Res.pdf](http://www.pocketdiagnostic.com/uploads/File/Datasheets/Phytophthora%20Data%20Sheet%20Hi%20Res.pdf) York, UK.

An antibody that recognizes the pathogen specific antigen is impregnated within the membrane of the test strip at two locations, one at the point of sample input and one further along the membrane at the positive (T line, see Fig. 1.5) site in the result window on the membrane cassette. The pathogen protein binds to the antibody which is bound to blue latex leads. As the solution moves along the membrane carrying the antibody/protein/latex combination it encounters a strip of

antibody/latex impregnated membrane at the test result site (T line) and binds further to these particles, giving a blue line as a positive test result. The more of the pathogen in the sample the stronger the blue positive line (T line) appears. If the sample does not contain the pathogen-specific protein, the blue latex beads do not move down the membrane and no positive blue line appears resulting in a negative test response. To ensure confidence in the test, an inert antibody moves within the solution to bind on a second test site (C line) to show that the membrane is functional. If the control line (C line) fails to turn blue it can be assumed that the test has failed and needs to be undertaken again with a new test kit (Anon., 2010b). Results appear in the viewing window of the test device approximately 5 min after starting the test. The result is easy to read, allowing the user to make a simple interpretation.

These Pocket Diagnostic kits allow growers, consultants and inspectors to diagnose diseases rapidly and in the field, with sampling and testing completed in a few minutes. For example, the *Phytophthora* Pocket Diagnostic kit is designed to be used widely across the horticultural industry and by statutory authorities for the detection of *Phytophthora* pathogens. The test can be used with woody material, for example to detect *Phytophthora* spp. in trees or with herbaceous material such as strawberry. Samples for testing can be taken from all parts of the plant (Anon., 2012b).

1.5. Biosensors for diagnosis of pea diseases

It would be beneficial if samples of leaf material could be used in a diagnostic kit in order to differentiate between pathogens that cause similar symptoms. For example, chlorosis is not only an early symptom of downy mildew infection of leaves but

also results from infection of root systems by the oomycete *Aphanomyces eutiches* (Gaulin *et al.*, 2007), the fungus *Fusarium solani* f. sp. *lisi* (Kraft and Boge, 2001) and nematodes (Inglis, 2001). Recent research has used proteomics to show that several proteins were altered in abundance as a result of *P. viciae* infection of pea leaves (Amey *et al.*, 2008). It has been suggested that such changes in protein may lead to identification of biomarkers that can be used in new techniques for diagnosis of disease (Amey and Spencer-Phillips, 2006).

Proteins are macromolecules that consist of amino acids, with the amino acid chain encoded by a RNA sequence which is transcribed from DNA. There are four levels of protein structure (Fig.1.6). The first is the primary structure, which is the sequence of amino acids that make up the polypeptides that comprise the protein. Secondary structure refers to the conformation of the protein chain, including helical and plated sheet structures. The tertiary structure is the three dimensional shape of the protein. Quaternary structure refers to the interaction of individual polypeptide chains, linked by covalent or non-covalent bonds, in proteins that consist of more than one polypeptide.

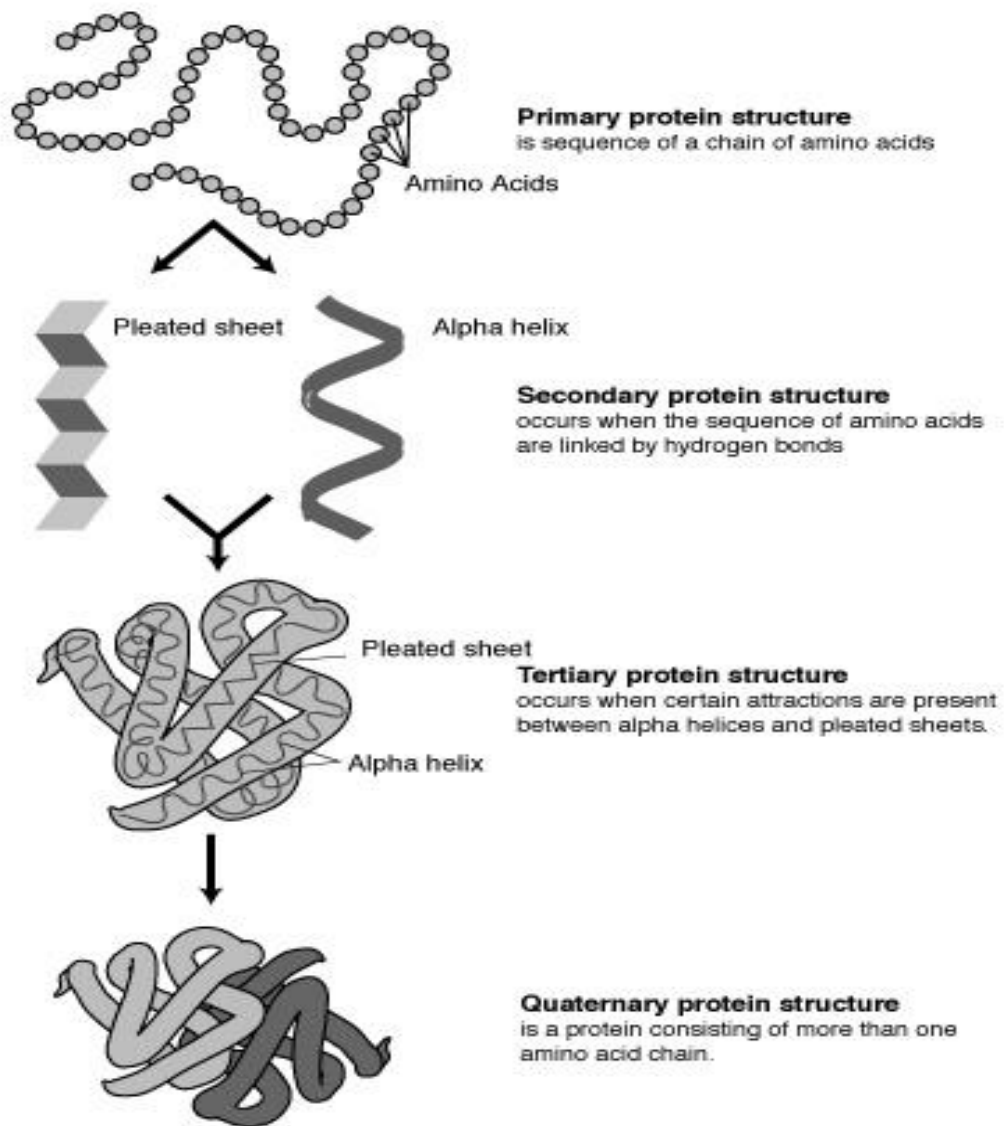


Figure 1.6. The four levels of protein structure from:

<http://matcmadison.edu/biotech/about/>

Proteomes have proved to be more variable in their properties than genomes, so there is no universal sample preparation method suitable for all proteins, and each source of protein presents its own sample preparation challenge (Hurkman and Tanaka, 1986). Therefore an early part of the present project has been to investigate different protein extraction methods, and to select the most appropriate for 2D-gel electrophoresis of proteins from pea roots.

1.6. Proteomics

The term proteome was first introduced in 1994 by Marc Wilkins (Dove, 1999), and refers to the proteins expressed in a given cell at a given time. Proteomics is defined as an attempt to characterize the biological state and other quantitative and qualitative changes of the protein content of cells (Tilg *et al.*, 2006). Proteomics includes several components such as protein separation, identification and quantification, protein sequence analysis, structural proteomics, interaction proteomics and protein modification. In its broader sense, proteomics involves protein activities, modifications, interactions and location in an organism or cell (Kavalliris and Marshall, 2005). It was considered to be a revolutionary technique because it promised to help understand the function of genomes. The discovery of the double helical structure of DNA (Watson and Crick 1953) and the development of techniques of DNA sequencing (Sanger *et al.* 1977) enable life science research to determine the genome sequence of living organisms. The complete genome sequences of several organisms including some plant species are available (Tabata, 2002; Frazier *et al.*, 2003). Thus the proteomics field promises to fill the gap between genome sequence and cellular behaviour (Dove, 1999). The rapid emergence of proteomics in biotechnology has been driven by the development, combination and automation of large scale analytical tools such as 1D and 2D gels and tandem mass spectrometry (Dove, 1999).

Currently there are three optimal methods for separation of complex proteins:

1. denaturing polyacrylamide (PAGE) also known as sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE), which was first reported by Laemmli, 1970;

2. 2D-gel electrophoresis, separates proteins based on their charge and molecular weight.

3. liquid chromatography (LC) which is a general term includes all forms of ion exchange, affinity and reversed-phase chromatography (Hunter *et al.*, 2002).

The 2D-gel electrophoresis method becomes an attractive method for the separation of complex protein samples and its impressive separation capabilities. In addition to that the 2D gel is usually preferred to LC-based approaches for protein separation because it is reproducible 2D gel proteome reference map that is a static, visual entity. An entirely annotated 2D gel reference map for a specific organ, tissue, cell, or organelle of interest is an important tool that can save time and money when land marking differentially expressed proteins in response to treatment, mutation or transgene introduction. Although 2D-gel electrophoresis suffers from well-publicized limitations, such as under-representation of membrane proteins (Wilkins *et al.*, 1998; Santoni *et al.*, 2000), 2D-gel electrophoresis and mass spectrometry are perhaps still the most important techniques for protein profiling and identification (Kav *et al.*, 2007). The application of proteomics in plant pathology has increased and is becoming very important with techniques such as 2D-gel electrophoresis and mass spectrometry being used to characterize cellular and extracellular virulence and pathogenicity factors produced by pathogens as well as to identify changes in protein levels in plant hosts upon infection by pathogenic organisms and symbiotic counterparts (Padliya and Cooper, 2006; Kav *et al.*, 2007). In addition, proteomics has been used to analyze various functional aspects of proteins such as post-translational modifications, protein-protein interactions, activities and structures (Park, 2004).

1.7. Early detection of pea root disease using infrared imaging

Photography is the process of recording visual images by capturing light rays on a light-sensitive recording medium for instance film or a digital charged-coupled device (CCD). Infra-red photography has been used for the detection of plant injury caused by different organisms (Heald *et al.*, 1972). It has potential for use as an advanced tool in diagnosis of plant disease. By this technique, the plant physiological state which is changed due to plant infection can be assessed at a distance from the plant tissue (Xu *et al.*, 2006).

Use of aerial photography to detect plant diseases was started from 1929 (Taubenhaus *et al.*, 1929), with Colwell (1956) used infrared for detecting plant diseases in cereal crops. Subsequently, Norman and Fritz (1965) used infrared sensitive colour film to detect the decline of Citrus trees in Florida and Meyer and French (1967) found that they could use Kodak Ektachrome Infrared (false-colour) film for detection of disease in forest and shade trees. In the same year, Manzer and Cooper (1967) stated that aerial photography could be used as a tool for basic potato disease research. It also became an important tool for the detection of infection and mortality of trees caused by root decaying pathogens (Williams, 1973).

Previous studies showed that the spongy mesophyll of plant leaves is the first part affected and starts to collapse before the green colour begins to fade when the plants undergo stress of different kinds of deficiencies, excesses, diseases and the infrared light was highly reflected as a result of these changes (Charter, 1959). Thus even though the plants looked normal to the human eye, they appeared different using infrared light (Charter, 1959).

In the present study, the effectiveness of infrared photography is assessed for diagnosis of pea root diseases in the early stages of infection and compared with manipulation of images captured using a standard digital camera.

1.8. Estimation of leaf area, fresh and dry weight of pea shoot and root system from plants infected by *F. solani* f. sp. *pisi* and *M. hapla*

Leaves in plants play two important roles. One role is photosynthesis, where chloroplasts trap light energy and change it to chemical energy which is used to fix CO₂. Products of photosynthesis from leaves are transported out via phloem to other plant parts (Weier *et al.*, 1982).

The other role is transpiration where the leaf structure is adapted to either conserve or release water vapour. Leaf area is a determinative factor in the amount of photosynthesis and therefore affects crop growth and yield potential. Measuring the leaf area is useful for understanding the relationship between leaf area and plant growth, and there is also a relationship between leaf area and fresh and dry weight (Aase, 1978; Cho *et al.*, 2007).

Roots are also important parts of plant systems as they provide anchorage and a surface for absorption of water and minerals, as well playing a role in nutrient storage. In healthy plants there is a balance between the root and shoot system, and between the parts of the plant which are exposed to the sun for manufacturing carbohydrates and the root surface which is in contact with the soil solution. Roots are required to supply a sufficient amount of water and other nutrients to the shoot system, and similarly sufficient carbon must be provided to the root system from the shoots (Weier *et al.*, 1982). Leaf and root growth is often affected by pathogen

infection, which is likely to be reflected in an altered proteome of tissue from infected plants.

1.9. Plant disease control

The aim of most plant disease control is to prevent or protect healthy plants and their products from diseases instead of curing after infection, in order to prevent or minimize the losses.

1.10. Methods of disease control

There are several methods of disease control, according to Franc (1998). Exclusion is a simple method of disease control, and includes several approaches such as quarantine, inspection, planting pathogen free materials, and seed stock certification.

Evasion is used as a protective procedure that uses disease-free seeds, and avoids disease through planting and growing at times that are unsuitable for disease development. In addition to selecting dates for planting and harvesting, this approach ensures that soil remains free of soil-borne pathogens.

Eradication involves the elimination of the pathogen within a defined area. It includes several ways of control such as host plant removal and destruction, treating soil and seeds with chemicals to kill the pathogen as well as treating equipment and stores.

A future method of control is environmental modification by creating an unsuitable climate for plant pathogens. Examples include reducing the humidity in greenhouses so it is not suitable for some pathogens, and reducing disease spread by increasing the spacing between plants. Soil irrigation and drainage are important

too. Soaking fields with water during the fallow time can reduce the incidence of some diseases caused by soil-borne pathogens. Environmental procedures used post-harvest include drying or refrigeration of harvested products.

Host resistance is deployed by using resistant varieties and is regarded as a safe, easy and environmentally desirable method for plant disease control. Indeed it is the only practical option available for control of some diseases, for example bacterial diseases where antibiotics are not available for use on crop plants.

Crop rotation is useful in disease control by growing plants that are not host to pathogens of previous crops. The period between the same crops depends on the period of survival of propagules of the pathogen. Crop rotation can give satisfactory control with soil-borne pathogens, but becomes less effective for pathogens that can survive for a long time in the soil (Agrios, 1997). In some cropping systems, the field is left fallow for a year or so, during which period microorganisms destroy the debris and inocula. In some hot summer areas, greater heating and drying of the soil occurs during a fallow period, which leads to reduction in soil-borne pathogens (Agrios, 1997).

Biological control is the destruction of pathogen populations totally or partially by other organisms that occur naturally in soil, and these organisms can be developed as biological control agents (Agrios, 1997). Although biological control is less effective and slow acting compared to chemical control, future improved performance is expected. Two commercial products of fungi that are predacious upon nematodes were prepared and marketed as Royal 300, containing the fungus *Arthrobotrys robusta* Duddington for controlling *Ditylenchus myceliophagus* Goodey on mushrooms and Royal 350 which is a different *Arthrobotrys* isolate for controlling *Meloidogyne* on tomato (Jatala, 1986).

The use of chemicals remains an important control measure, despite concerns about their impact on human health and the environment. Protection prophylaxis is the preferred method to reduce plant infection. Correct timing of use of these chemicals and choosing the proper chemical is very important for good control. Several groups of chemicals are used to control plant diseases, some of which have a general broad target range, whilst others are specific (Franc, 1998). For example, fumigants and sterilants have a wide activity range on all living organisms include growing plants. Soil fumigations are usually used to reduce soil-borne pathogens, in addition to plant parasitic nematodes and other pests. These types of chemicals have many disadvantages such as being expensive, highly toxic, non-selective, and difficult to apply. Nematicides are typically applied as liquids and granules, and can be added to soil before and after growing plants. They typically kill nematodes and insects, and most nematicides are highly toxic, and can contaminate ground water if not used properly (Franc, 1998).

Seed treatment usually includes using fungicides to protect seeds and seedlings from seed-borne and soil-borne infection. An advantage of seed treatment is that relatively small amounts of chemicals are used (Franc, 1998). Whilst protectant fungicides are applied to aerial parts of the plant and remain on the surface to prevent infection (Franc, 1998), systemic fungicides are absorbed by the plant tissue and translocated via the plant vascular system. Their effect is in killing or suppressing the plant pathogen. They may have a curative or therapeutic affect and often are specific to particular groups of fungi and oomycetes (Franc, 1998).

Control is best provided using integrated methods. Integrated control has advantages such as cost reduction and a decrease in other risks that might be associated with one single control measure. Integrated pest management (IPM) is

defined as a "sustainable approach to managing pests by combining biological, cultural, physical and chemical tools in a way of minimizes economic, health, and environmental risk" (Knodel and McMullen, 1999).

According to Bird (1987), IPM includes seven components: biological monitoring, environmental monitoring, the decision maker, decision support systems, the decision procedure, procedure implementation, the system. IPM applied for nematode control will become imperative because of human health and environmental concerns following increased use of nematicides in modern agriculture.

Currently most nematode management strategies and tactics include exclusion or avoidance, reducing initial population density, suppressing nematode reproduction and restricting current crop damage (Barker, 1997). An effective chemical control method used previously was to fumigate soil with methyl bromide, but this was phased-out under the Montreal Protocol on substances since January 2005, because of its ability to destroy the Ozone layer. Methyl bromide use is now restricted to nematode infected fields in fruit and nut nurseries in California, because the Montreal protocol allows for Critical Use Exemption (CUE), where there is no effective alternative for controlling plant parasitic nematodes (Zasada *et al.*, 2010).

Before appropriate control measures can be applied, however, the cause of disease symptoms must be determined. As nematode infection causes very similar symptoms on aerial parts of plants to those caused by other root-infecting pathogens, a rapid diagnostic kit to distinguish the different causes would be valuable. Proteomic comparison of leaves from healthy and infected plants has the potential to identify protein biomarkers that could be used, for example, in pocket diagnostic kits for rapid and cheap identification in the field (Amey and Spencer-

Phillips, 2006; Padliya and Cooper, 2006). Analysis of the proteins will also provide information about the molecular causes of symptoms and host response to infection (Bhadauria *et al.*, 2010).

1.11. Aims of the project

Proteomics has become a powerful tool with potential to identify plant pathogens and to understand the interaction between plant pathogens and their hosts. Proteomics is still a relatively new field of study and proteomic studies of pea plants are limited. Thus the hypothesis of this study is that proteomics can be used for the analysis and identification of proteins showing changes in their abundance as a result of infection of pea plants by *P. viciae*, *F. solani* and *M. hapla*, and that digital imaging techniques have potential for studying changes in pea leaves as a result of infection by root pathogens (*F. solani* and *M. hapla*).

For these purposes, the specific objectives were to:

1. establish appropriate methods for extracting proteins from pea roots and leaves;
2. study the responses of the pea plant proteome to infection by downy mildew, root knot nematode and *Fusarium* root rot pathogens;
3. determine whether leaf infection is reflected in an altered proteome of roots and whether root infection modifies the leaf proteome;
4. determine whether the amount of protein in pea roots and leaves is altered as a result of these diseases;
5. determine whether the size of the root system and leaf area is affected by these infections;
6. identify possible protein biomarkers that could be used in diagnosis of these diseases;
7. use digital visible light and infra-red imaging of leaves in diagnosis of these diseases in pea.

Thus this study aims to use proteomic and imaging techniques to understand the interaction between pea plant and *P. viciae*, *F. solani* and *M. hapla*, particularly in relation to rapid, non-destructive and in-field diagnosis of disease.

2. Materials and Methods

2.1. *Pisum sativum* L. (pea) cultivars

Seeds of six cultivars of *P. sativum* L. (Table 2.1) were germinated on filter paper soaked in distilled water for 24 h at room temperature. Then these seeds were sown in pots (10 cm diameter) containing Levingtons f2 compost with four seedlings per pot, and the pots were incubated at 20°C in a cycle of 16 h of light and 8 h of dark.

Table 2.1. Cultivars of pea (*Pisum sativum* L.) used for inoculation with *Peronospora viciae*, *Meloidogyne hapla* and *Fusarium solani*.

No.	Cultivars	Source
1	Livioletta	Unwins Ltd, UK
2	Solara	Unwins Ltd, UK
3	Kelvedon Wonder	Booker Seeds Ltd, UK
4	Krupp Pelushka	Deutsche Saatveredlung, Germany
5	Early Onward	Booker Seeds Ltd, UK
6	Maro	Unwins Ltd, UK

2.2. *Solanum lycopersicon* (tomato) cultivars

Seeds of six tomato cultivars were sown in pots (10 cm diameter) containing autoclaved top soil and incubated in a regime of 16 h of light and 8 h of dark and 20°C for maintaining *Meloidogyne hapla*. The soil was autoclaved in batches of approximately 2 kg at 121°C and 1.06 kg/cm² for 15 min.

Table 2.2. Cultivars of tomato (*Solanum lycopersicum* L.) used for maintaining *Meloidogyne hapla*.

No.	Cultivars	Source
1	Cuor di Bue	Suttons, England
2	Gardener's Delight	B & Q, UK
3	Money Maker	B & Q, UK
4	Alisa Craig	B & Q, UK
5	Zuccherò	B & Q, UK
6	Marmonde	B & Q, UK

2.3. Inoculation of pea plants with *Peronospora viciae* f. sp. *pisi*

The source of the *Peronospora viciae* f. sp. *pisi* inoculum was infected pea plants kindly supplied by Dr Jane Thomas from the National Institute of Agriculture Botany (NIAB, Cambridge). To maintain the inoculum, six pea cultivars (Table 2.1) were inoculated using conidiospores (referred to subsequently as conidia) from a stock which had been stored on intact leaves at -80°C. To prepare the inoculum, infected leaves were shaken in distilled water to obtain conidia, and then the suspension was filtered through a 45 µm mesh to remove debris. The density of spores was measured using a haemocytometer and the suspension was diluted with distilled water to a final density of 5×10^5 spores/ml.

2.3.1. Leaf inoculation

To inoculate pea leaves, the wax layer was flattened by brushing the upper surface of leaves gently with a fine paint brush to enable better adhesion of the conidia. The

suspension of spores was sprayed on the leaves using a hand-held garden sprayer. After inoculation, plants were kept in darkness and in high humidity by covering them with a plastic sheet for 24 h at 14°C. After this period, plants were left for another 24 h at 14°C with 16 h light and 8 h darkness. The plastic sheet was removed 48 h after inoculation and the plants left at 20°C under a regime of 16 h light and 8 h dark for 6-7 days. To induce sporulation, the infected plants were covered as described above with incubation at 14°C with 16 h light and 8 h darkness for 4-8 days.

2.4. Inoculation of pea plants with *Fusarium solani* f. sp. *pisi*

The source of inoculum was *Fusarium solani* f. sp. *pisi* supplied by CABI Europe (UK, Egham), as a freeze dried culture, and maintained by growing on Potato Dextrose Agar (PDA). Two inoculation methods were used to inoculate pea plants.

2.4.1. Soil inoculation method of Clarkson (1978)

Czapek-Dox Yeast (CDY) liquid medium (Oxoid, UK) was prepared by dissolving 39 g in 1000 ml SDW and autoclaved at 121°C for 15 min. Aliquots of 20 ml were poured into plastic Petri dishes which were inoculated with *F. solani* by transferring a small piece of mycelium taken with a sterile mounted needle from a stock culture grown on potato dextrose agar (PDA). Inoculated Petri dishes were incubated at room temperature for five days. Top soil was autoclaved twice in approximately 2 kg batches at 121°C and 1.06 kg/cm² then the moisture of the soil was decreased by drying it at room temperature. Mycelia and conidia were added to the cooled soil at a rate of 20 ml per 337g soil, and mixed to give an even distribution. The soil was placed in 10 cm pots, then surface-sterilized seeds (25% chlorax for 2 min, washed

in sterile distilled water for 2 min) were sown in the pots. Pots were then incubated at 20°C in a cycle of 16 h of light and 8 h of dark.

2.4.2. Seed inoculation method of Kraft and Kaiser (1993)

This inoculation method was based on the method of Kraft and Kaiser (1993) as modified by Ondrej *et al.* (2008). Pea seeds were surface sterilized as above then soaked in deionised water for 24 h, before soaking overnight in a conidial suspension of *F. solani* adjusted to 1×10^6 spores/ml. Inoculated seeds were sown in pots which were filled with sterilized soil as above. The plants were watered regularly and incubated for 4-5 weeks at 20°C in a cycle of 16 h of light and 8 h of dark.

2.5. Inoculation of pea plants with *Meloidogyne hapla*

The source of inoculum was *M. hapla* eggs which were kindly supplied by Dr Valerie Moroz Williamson, Department of Nematology, University of California Davis. The nematode was maintained on several tomato cultivars (Table 2.2), and the method of inoculation was the same as described below for pea. The nematode eggs were extracted using the Hussey and Barker (1973) method, where infected roots were cut into approximately 1-2 cm long segments, and then put in a 500 ml jar containing 0.5% NaOCl. The jar was shaken vigorously by hand for 2-3 min, and then the contents were passed quickly through a 200 mesh (75 µm pore size) sieve to remove the plant debris. The suspension was passed through a 500 mesh (25 µm pore size) sieve to collect eggs, and the 500 mesh sieve plus eggs was washed under a stream of cold tap water for approximately 3 min to remove the NaOCl residues. Finally, the eggs were collected in a clean beaker, the numbers of

eggs determined using a Chambered counting slide (Chalex Corporation, USA), and then the density adjusted using a measuring cylinder by adding water to give 2000 eggs and juveniles at development stage J2/ml. To inoculate pea plants a 25 ml aliquot of the suspension of *M. hapla* eggs was injected into soil near the roots using a pipette. The plants were grown in pots sown with 4 seeds of *Pisum sativum* cv Livioletta, and after 4 days the seedlings were thinned to one per pot, and were at the two leaf stage of development (Stephane, 1983; Udo *et al.*, 2005; Anita *et al.*, 2006). Inoculated plants were incubated at 20°C in a regime of 16 h light and 8 h dark, until symptoms started to appear on infected plants (typically 28 days after inoculation).

2.6. Methods of protein extraction

Several methods were used to extract proteins from pea leaves and roots.

2.6.1. Pea roots

2.6.1.1. Amey method (Chuisseu *et al.*, 2007)

Pea roots (approximately 100 mg) were ground in liquid nitrogen and resuspended in 10 volumes (1000 µl for 100 mg) of lysis buffer containing 25 mM Tris (pH 8.5), 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 7 M urea and 2 M thiourea. The homogenate was vortexed for 5 s and shaken for 1 h at 4°C. The suspension was then centrifuged for 1 h at 22,000 g at 4°C and the supernatant stored at -80°C.

2.6.1.2. Brigham method (Brigham *et al.*, 1995)

Pea root tips (2.5 cm; approximately 100 mg) were ground in liquid nitrogen, then homogenized in 10 volumes (as above) SDS extraction buffer (4% sodium dodecyl sulfate (SDS), 5% 2-mercaptoethanol, 5% sucrose) as described by Colas des Francs *et al.* (1985). The homogenate was centrifuged for 20 min at 10,000 g. Proteins were precipitated from the supernatant with acetone overnight at -20°C and then resuspended in sample buffer (60 mM Tris-HCL, pH 8.8; 60 mM dithiothreitol (DTT); 2% (SDS); 15% sucrose; 5 mM amino-N-caproic acid; 1 mM benzamidine; 0.01% bromophenol blue) and stored at -20°C.

2.6.1.3. SDS and SDS-acetone methods (Shultz *et al.*, 2005)

This method included two different extraction procedures. In each procedure, 200 mg of pea roots were ground briefly in liquid nitrogen and the extractions were prepared as follows:

(1) SDS method. A 500 µl volume of SDS-PAGE loading buffer (2% SDS; 60 mM Tris, pH 6.8; 10% glycerol; 100 mM DTT) was added to the root tissue powder and incubated on ice for 15 min, and then centrifuged at 16000 g for 10 min at room temperature. In the last step, the supernatant containing SDS-extracted protein was transferred to a new tube and stored at -80°C.

(2) SDS-acetone method. A 500 µl volume of SDS extracted sample (see above) was precipitated by incubating for 1 h at -20°C in 2 ml acetone solution containing 0.07% 2-β mercaptoethanol, then centrifuged at 16,000 g for 30 min at 4°C. Next, the protein pellet was washed three times with 1 ml of acetone. During each wash step the pellet was dispersed by sonication for 15 s and the samples were

centrifuged at 16,000 g for 5 min at 4°C. The final pellet was dried at room temperature for 5 min and resuspended in 500 µl of SDS-PAGE loading buffer.

2.6.1.4. TCA-acetone method (Natarajan *et al.*, 2005)

This protocol was modified by Shultz *et al.* (2005) from the original Natarajan *et al.* (2005) method, and involves extraction in a solution containing TCA and acetone. A 200 mg sample of root tissue powder was dissolved in 1 ml of TCA-acetone extraction solution (90% acetone, 10% TCA, 0.07% β-mercaptoethanol), the sample was vortexed and incubated at -20°C for 1 h, then centrifuged at 22,000 g for 1 h at 4°C. The protein pellet was washed by centrifugation three times with acetone and sonicated during each step for 15 s. The pellet from the last step was dried at room temperature for 5 min then resuspended in 500 µl of SDS-PAGE loading buffer.

2.6.1.5. BPP method (Wang *et al.*, 2007)

In this protocol, the extraction procedure was modified by Wang *et al.* (2007) from a previously published protocol (Saravanan and Rose, 2004), and was referred as the borax/PVPP/Phe (BPP) method. In short, 100 mg of tissue were ground briefly in liquid nitrogen using a mortar and pestle, and then the powder dissolved in 1000 µl of lysis buffer, prepared as in the Amey method (above) but also containing 1% polyvinylpolypyrrolidone (PVPP) to inhibit activation of proteolytic enzymes and to remove interfering compounds.

2.6.2. Pea leaves

2.6.2.1. Giavalisco method (Giavalisco *et al.*, 2003)

Proteins were extracted from leaves of healthy pea plants and plants infected by *F. solani* f. sp. *pisi* and *M. hapla* using the method of Giavalisco *et al.* (2003). This method is claimed to extract three groups of protein: 1) cytosolic proteins; 2) membrane bound proteins; 3) nucleic-associated proteins. A 500 mg fresh weight batch of pea leaves was ground in liquid nitrogen using a mortar and pestle, then 62.5 µl of 0.125% (v/w) inhibitor mixture 1 (100 mM KCl; 20% v/v glycerol; 50 mM Tris pH 7.1) plus a Complete Protease Inhibitor Cocktail Tablet (Roche, Germany), and 0.05% (w/w) of inhibitor mixture 2 (1 mM pepstatin A, 1.4 mM phenylmethylsulfonylfluoride (PMSF) were added. The samples were centrifuged for 60 min at 22,000 g at 4°C. The supernatant, which contains the water-soluble cytosolic protein (fraction 1), was removed and 54 mg urea, 5 µl of 700 mM fresh DTT and 5 µl of Ampholyte IPG buffer (pH 3-10), (Sigma, UK) were added to every 50 µl. The supernatant was either analyzed immediately or stored at -80°C.

The pellet was ground in liquid nitrogen, then 0.125% (w/w) of inhibitor mixture 3 (200 mM KCl; 20% v/v glycerol; 100 mM phosphate buffer, pH 7.1) and Complete Protease Inhibitor Cocktail (as above) was added together with one volume of buffer A (100 mM phosphate buffer, pH 7.1; 200 mM KCL; 20% v/v glycerol; 2 mM MgSO₄; 4% CHAPS) in addition to 2% (w/w) amidosulfobetaine-14,3-[N,N-Dimethyl (3-myristoylamino)propyl] ammonio] propanosulfonate (ASB 14) detergent (Calbiochem, UK). Next, 0.025% (v/w) DNase (Sigma, UK) was added to the samples and they were homogenised thoroughly and mixed by stirring at 4°C for 45 min. After that 23% v/w of buffer B (700 mM DTT, 7 M urea, and 2 M thiourea) was added to the samples and they were stirred at room temperature for 45 min.

Then the homogenate was centrifuged for 60 min at 22,000 g at 17°C. The supernatant which contains membrane protein and nucleic acid-associated proteins was removed, 5 µl of ampholyte IPG buffer (pH 3-10) (Sigma, UK) was added, and the protein stored at -80⁰ C ready for 2D-gel electrophoresis.

If necessary the 2D Clean-up Kit (Amersham Biosciences) was used to clean the protein (see below), and the protein was resuspended in lysis buffer (30 mM Tris; 4% (v/v) CHAPS; 7 M urea; 2 M thiourea were dissolved in 50 ml sterile deionised water and then adjusted to pH 8.5). The protein was quantified using the 2D Quant kit (Amersham Biosciences) according to the manufacturer's protocol as shown in section 2.9.

2.6.2.2. Amey method (Chuisseu *et al.*, 2007)

Approximately 500 mg of pea leaves were ground in liquid nitrogen and resuspended in 10 volumes of lysis buffer as described for pea roots in section 2.6.1.1 (above).

2.7. Conductivity

As a result of salt problems that affect the running of 2D-gel electrophoresis (see below), several experiments were conducted to measure the amount of salt in protein solutions extracted from roots. A conductivity meter was calibrated using 100 mM NaCl and the conductivity of NaCl solutions prepared at concentrations of 50, 100, 150 mM were also measured, for comparison with measurements of conductivity of protein extracts.

2.8. Protein desalting

Several problems can affect the integrity of the isoelectric focusing gel as a result of high salt concentration, leading to loss of current. The ions which affect the strips can come from either the samples or the buffer. In addition to that there are several contaminants, such as lipids, ionic detergents, nucleic acids, polysaccharides, phenolic compounds and insoluble materials which can affect the strips (Jefferies, 2008). To overcome these difficulties, a number of techniques can be used. A 2D Clean-up Kit (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) was used for protein de-salting, and Zeba De-salt Spin Columns (Pierce, Rockford, UK) were used for protein de-salting and recovery.

2.8.1. 2D Clean-up Kit

A 300 μ l volume of precipitant solution from the 2D Clean-up Kit was added to a 100 μ l sample containing 100 μ g of protein, then vortexed and incubated on ice for 15 min before adding 300 μ l of co-precipitant solution and mixing. Following centrifugation at 12,000 g for 5 min, the supernatant was removed and 40 μ l of co-precipitant was added. The tubes were kept on ice for 5 min, centrifuged again at 12,000 g for 5 min, and then 25 μ l of deionised water was added to the protein pellet which was dispersed by vortexing for 10 s. Next, 1 ml of chilled washing buffer and 5 μ l of wash additive were added and vortexed for 30 s every 10 min for 30 min. Finally, the preparation was centrifuged at 12,000 g for 5 min, the supernatant removed and the pellet allowed to dry for 5 min, before it was resuspended in 100 μ l of sample solution.

2.8.2. Zeba De-salt Spin Column

This method involves both de-salting and protein recovery, and also is very quick compared with the 2D Clean-up Kit method. The latter takes a relatively long time (approx.1.5 h) and much protein is lost during the procedure. To prepare a spin column, the base was removed and the column placed in a 1.5 ml Eppendorf tube. It was then centrifuged at 6,000 g for 1 min to remove the storage solution. The top of the compacted resin was then marked and the column was placed in a new Eppendorf tube. The cap was removed from the column and 50-130 μ l of protein solution were added. This was centrifuged at 6,000 g for 2 min, then the column was discarded and the Eppendorf tube containing the protein solution was kept. The process was repeated with a further batch of protein solution until all of the extract was purified and all protein solutions were combined in one tube.

2.9. Protein quantification (Bradford method)

There are several methods to determine the protein concentration in a sample, based on the binding of various dyes to the protein. This can be done either by staining the protein in suspension and then measuring the absorption spectrophotometrically, or by staining the protein bands after electrophoretic separation on sodium dodecyl sulphate (SDS) gel. The simplest method is the Coomassie brilliant blue method of Bradford (1976). This procedure is suitable for detection of 1.0 μ g of protein and is fast, requiring only one reagent, and the colour intensity is stable over a period of 1 h. A protein standard stock solution was prepared with Bovine Serum Albumin (BSA) to give a concentration of 1 mg/ml in double distilled water, and this was used at a range of dilutions to give a protein standard curve. A 1 ml aliquot of Bradford reagent (Sigma-Aldrich, UK) was added to 100 μ l of each dilution, then

mixed well and allowed to stand for 5 min at room temperature before absorption was determined at 595 nm. A graph of BSA standard protein content (μg) versus absorption was plotted, from which the protein content of the extract samples were determined.

2.10. 1D-gel electrophoresis (SDS PAGE)

The separation of macromolecules such as proteins in an electric field is known as electrophoresis. In the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) method, which is the most commonly used low-level protein separation and purification technique in biological research, acrylamide gel is used as a support medium and SDS is used to denature the proteins. In the method used in the present work, casting stands are used to prepare mini-slab gels. Proteins were separated according to their molecular weight on a 12% gel which consists of two layers. The lower layer is known as the running gel and was prepared by mixing 2.7 ml bis- acrylamide (Sigma, UK), 2.3 ml of 1.5 mM Tris-HCl (pH 8.8), 90 μl of 10% SDS, 3.1 ml distilled water, 50 μl of 10% ammonium persulphate (APS), and 5 μl of tetramethylethylenediamine (TEMED).

These solutions were mixed together in a 50 ml tube and poured into a mini gel system (Atto Corporation, Japan; Fig. 2.1), then left for 1 h at room temperature. The upper layer is known as the stacking gel and was prepared by mixing 0.375 μl of 30% acrylamide, 1 ml of 1.5 mM Tris-HCl (pH 8.8), 40 μl of 10% SDS, 50 μl of 10% APS, and 10 μl of TEMED.

The stacking gel was poured into the mini gel system and left for half an hour at room temperature.

Protein solutions were loaded in each gel chamber using a pipette. After electrophoresis, the gel was stained with colloidal Coomassie stain (Sigma) and then scanned using an image scanner (Amersham, Buckinghamshire, England) with version 5.0 labscan software (Amersham), and then the images were saved in TIF format.

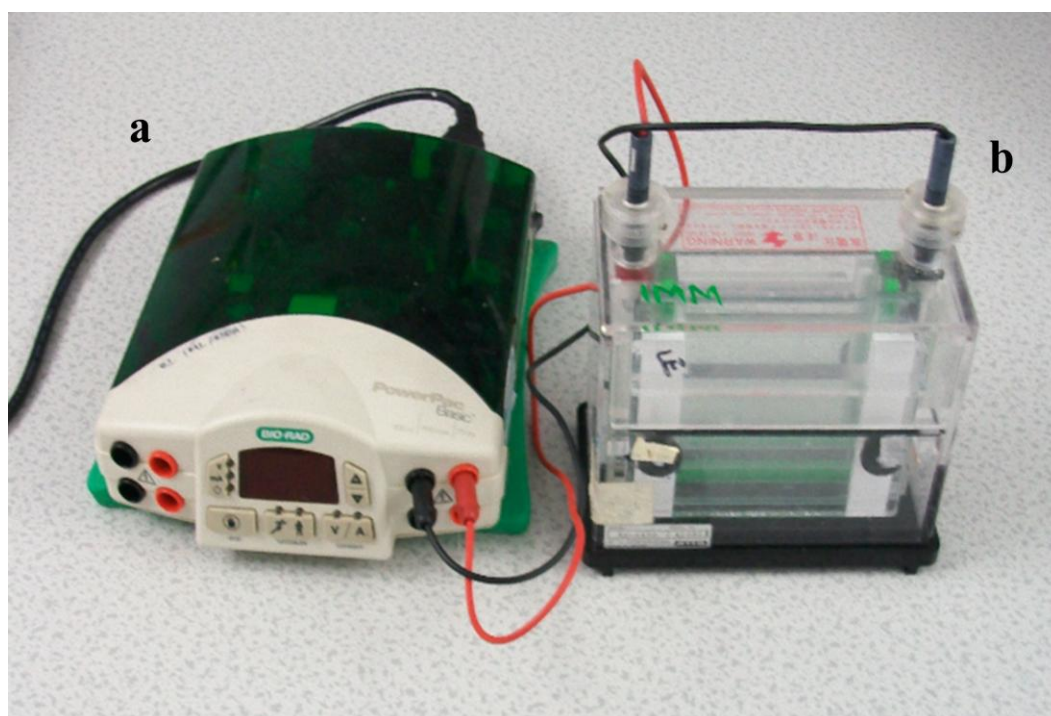


Figure 2.1. Mini gel system (Atto Corporation, Japan)

- a) Electrophoresis power supply.
- b) Electrophoresis chamber.

2.11. 2D-gel electrophoresis

2D-gel electrophoresis has been the most popular separation technique used in proteomics (Dove, 1999), and separates proteins in two steps. The first separates according to the isoelectric point (PI) in a step called isoelectric focusing (IEF). The

second step separates proteins according to their molecular weight in a process called SDS-polyacrylamide gel electrophoresis (O' Farrell, 1975).

2.11.1. Isoelectric focusing

In this method, 24 cm immobilised pH gradient (IPG) strips with a non-linear pH of 3-10 (GE Healthcare) were rehydrated with the protein solution (300 µl protein extract and 150 µl of rehydration buffer containing; 8 M urea, 2% CHAPS; 0.002% bromophenol blue; 0.28% DTT and 2% IPG carrier ampholyte. The strip holders (Fig. 2.2a) were cleaned with drops of strip holder cleaning solution (GE Healthcare) and a toothbrush, and then were rinsed with sterile distilled water (SDW). The strips were removed from their packet using clean forceps and by holding only at the ends of the strips. The protective covers of the strips were removed using another pair of forceps, and the strips were positioned with the acidic (+) end towards the pointed ends of the holder, and were lowered into the protein solution already placed in the 24 cm Ettan IPG-phor strip holder (GE Healthcare) to ensure that the solution was distributed along the length of the strip. For each strip, 3 ml of Immobiline dry strip cover fluid (Amersham Biosciences) was pipetted to overlay each strip. This amount was distributed along the strip holder by pipetting 1 ml each end and 1 ml in the middle, then distributed evenly until the liquids met each other. Finally the cover was placed onto the strip holder to ensure the strips made perfect contact with the electrode, before they were put in an IPG-phor unit (GE Healthcare) (Fig. 2.2b) and subjected to 500 V for 1 h, 1000 V for 1 h and 8000 V for 8 h 20 min (all at 20°C) for isoelectric focusing. The strips were either equilibrated directly (see below) and used for second dimension electrophoresis or stored at -80°C for using another time.

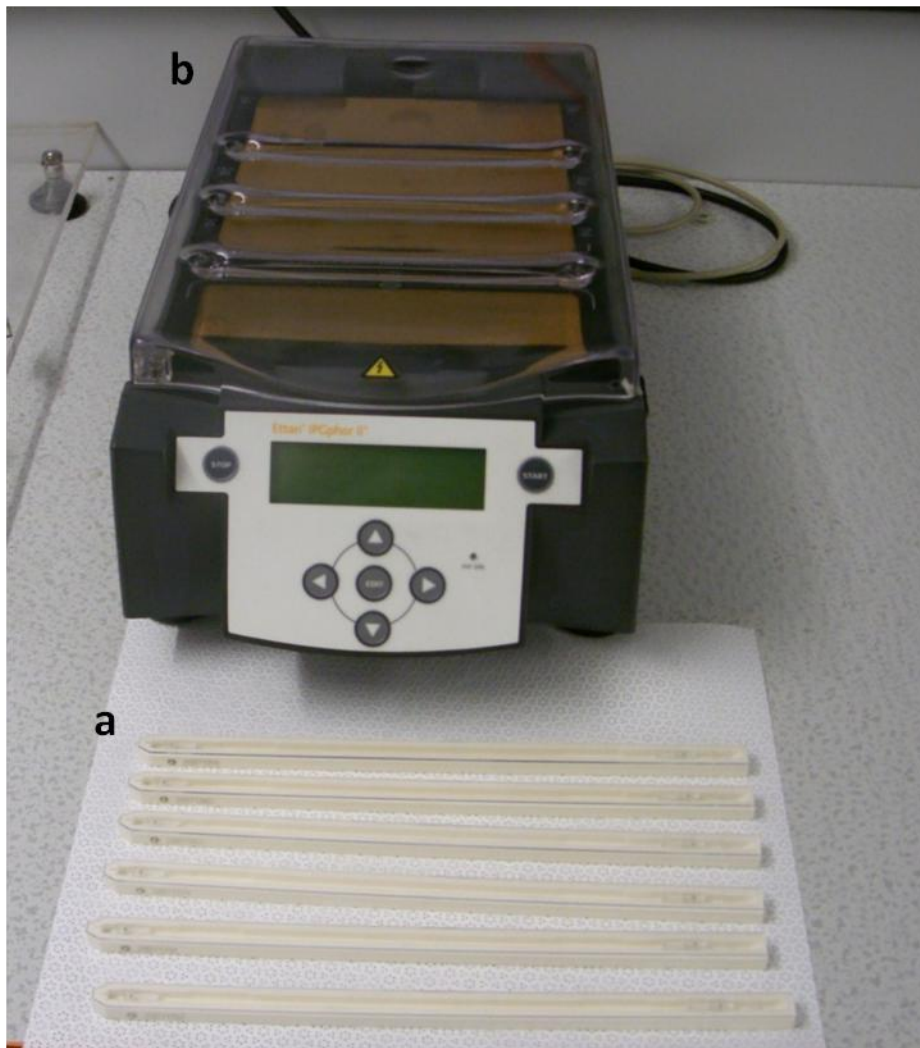


Figure 2.2. Six strip holders (a) and Ettan IPG-Phor system (b) (GE Healthcare).

2.11.2. IPG-strip equilibration

IPG strips stored at -80°C were taken out of the freezer, thawed at room temperature then equilibrated twice for 15 min in two different equilibration buffers. The first equilibration buffer comprised: 50 mM Tris-HCl, pH 8.8; 6 M urea; 30% v/v glycerol; 2% w/v SDS; 0.025% w/v bromophenol blue containing

1% w/v DTT. The second equilibration buffer was the same as the first but with 2.5% (w/v) iodoacetamide instead of DTT, and its role was to remove excess DTT. The tubes containing the strips and second buffers were protected from light using aluminium foil, because the iodoacetamide is sensitive to light. Finally, the equilibrated IPG gel strips were rinsed gently once with SDS electrophoresis buffer (25mM Tris-HCl, 192 glycine, 0.1 SDS, pH 8.3) to remove excess equilibration buffer and then applied onto the second dimension gel.

2.11.3. Preparing the Ettan DALT six gel casters (GE Healthcare, Buckinghamshire, England)

The triangular rubber seal was set at the bottom of the gel caster (Fig. 2.3). The gel cassettes were cleaned with sterile distilled water (SDW) and then 70% ethanol to remove any dust particles that might be present on the glass surface. The caster was loaded with a separator sheet against the back wall and further separator sheets were placed between gel cassettes, as they were loaded. Blank cassettes were loaded, if not using all six spaces. Six separator (1 mm thick) sheets were placed at the end of the caster. The gasket was put into the groove of the face plate of the caster after lubricating with a light coating of gel sealant. Finally the face plate was placed onto the gel caster and tightened with six spring clamps and two screws, then the caster was ready for pouring the gel.

To prepare the gel, 12.5% acrylamide was prepared using a 40% acrylamide solution by mixing 62.5 ml of acrylamide with 50 ml of resolving gel buffer (1.5 Tris-HCl, pH 8.8), 85.3 ml of SDW and 2 ml of 10% SDS. This mixture was filtered through a 45 µm pore filter paper to de-gas before 1 ml of APS and 66 µl of TEMED were added. The acrylamide solution was poured immediately into the gel

caster through the filling channel, leaving a 1 cm space below the edge of the short plate. After that, 2-3 ml of either 30% isopropanol or 1% SDS was pipetted on the top of the gel, and then the caster was covered with a wet paper sheet and plastic sheet to maintain the humidity and left overnight to set.

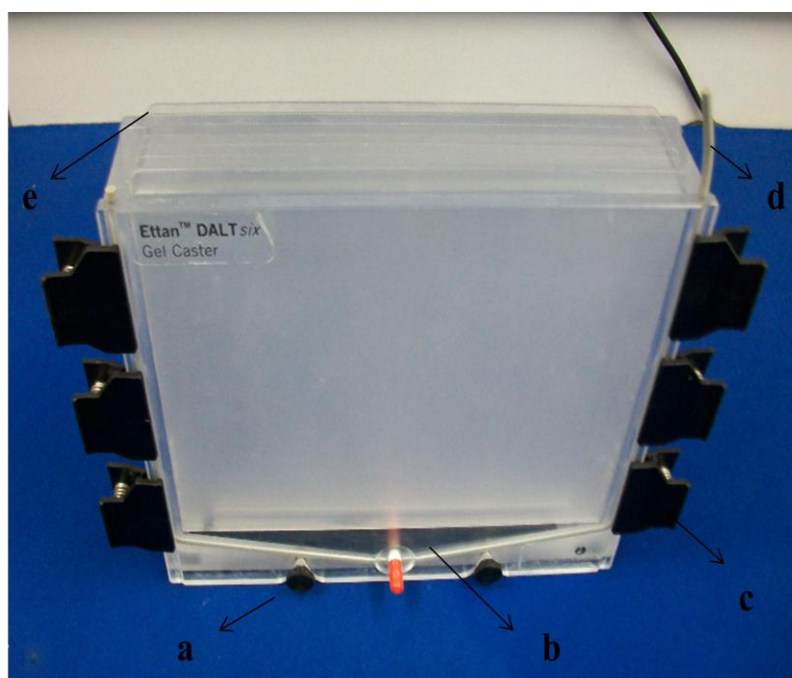


Figure 2.3. Ettan Dalt Six Gel Caster (a) Screw, (b) triangular rubber, (c) spring clamp, (d) gasket, (e) separator sheet.

2.11.4. Unloading the gel caster

The face plate was taken off after removing the screws and the spring clamps, then the cassettes were taken out carefully by pulling the separator sheets plus cassettes forward. The top surface of each gel was rinsed with SDW, then the gels were either used immediately or stored at 4°C after they were wrapped with Saran wrap (SC Johnson) or aluminium foil.

2.11.5. Second dimension

In this step, protein was separated due to its molecular weight. The equilibrated strips were placed on the top of a 12.5% SDS-polyacrylamide gel (40% acrylamide solution, 1.5 mM Tris-HCl, pH 8.5, plus 10% sodium dodecyl sulphate in distilled water that was filtered through a 0.45 µm pore filter paper for de-gassing) then 10% fresh APS and TEMED were added. A piece of filter paper was impregnated with 5 µl of protein molecular weight standard (Biorad) and placed directly on the gel surface at the left hand side of the IPG strip. The cassettes were laid on a flat surface with the short glass plate face up, and the strips were rinsed once with SDS electrophoresis buffer (25 mM Tris-HCl, 192 mM glycine, 0.1 SDS, pH 8.3) before being taken out using clean forceps and centred on the long glass plate with the acidic end placed to the left. The strips were manipulated using small metal spatula until they made complete contact with the gel surface so that bubbles were avoided. Agarose sealing gel (0.5% agarose, 0.002% bromophenol blue in SDS electrophoresis buffer) (25mM Tris-HCl, 192 mM glycine, 0.1 SDS, pH 8.3) was melted in a microwave oven and slowly pipetted along the upper surface of the IPG strips.

The electrophoresis unit (Fig. 2.4) was prepared by inserting the anode assembly into the tank, and was filled with SDS electrophoresis buffer (25mM Tris-HCl, 192 mM glycine, 0.1 SDS, pH 8.3). The pump was switched on and the temperature was set at 10°C using a Multitemp III cooling unit (Amersham), then the gels and blank cassettes were inserted. The Lower Buffer Chamber (LBC) was filled with SDS electrophoresis buffer (25mM Tris-HCl, 192 mM glycine, 0.1 SDS, pH 8.3), while the Upper Buffer Chamber (UBC) was filled with SDS electrophoresis buffer (50 mM Tris-HCl, 364 mM glycine, 0.2 SDS, pH 8.3). The lid was placed in position

and electrophoresis begun at 10°C (Eps-601 power supply at 2.5 W per gel for 30 min, subsequently increased to 18 W per gel for 5 h). When the bromophenol blue from the agarose sealing gel reached to the bottom of the gel, the power supply and cooling unit were turned off, the lid was removed from the electrophoresis tank, followed by the UBC. The gel cassettes were removed and were opened by twisting the plastic wedge tool between the two glass plates. Each gel was marked with a cut to the bottom right hand corner, which helped to identify the gel and its correct orientation after making a note of the gel number using the number of the IPG strip at the top of the gel. The gels were put in suitable trays in fixing solution and covered by Saran wrap or aluminium foil. The fixing stage was very important as it immobilized the separated proteins in the gel and removed any non-protein components which interfered with subsequent staining.

The gels were fixed by incubating in 10% methanol, 7% acetic acid for 1h with very gentle shaking using an orbital shaker (Gyro-Rocker, SSL3). After replacing the fixing solution, the gels were submerged overnight in Coomassie blue. This was prepared by adding 800 ml of deionised water to the bottle labelled Brilliant Blue G-Colloidal concentrate and the working solution stored at 4°C once diluted. Before using this solution, 4 parts of the working solution were combined with 1 part of methanol and mixed well for 30 s. After overnight staining, gels were destained for 60 s using 10% acetic acid in 25% v/v methanol with gentle shaking as before. Finally the gels were rinsed briefly using 25% methanol, then destained in fresh 25% methanol for up to 24 h to remove any precipitated dyes from the gel surface. A clean piece of cotton wool or a lab wipe soaked in 25% methanol was used for removing any remaining spots of dye. The gels were scanned using an

image scanner (Amersham, Buckinghamshire, England) with version 5.0 labscan software (Amersham Biosciences), then the images were saved in TIF format.

The captured images were analysed using advanced PDQuest-Analysis software version 8.0 (Bio-Rad laboratories, USA).

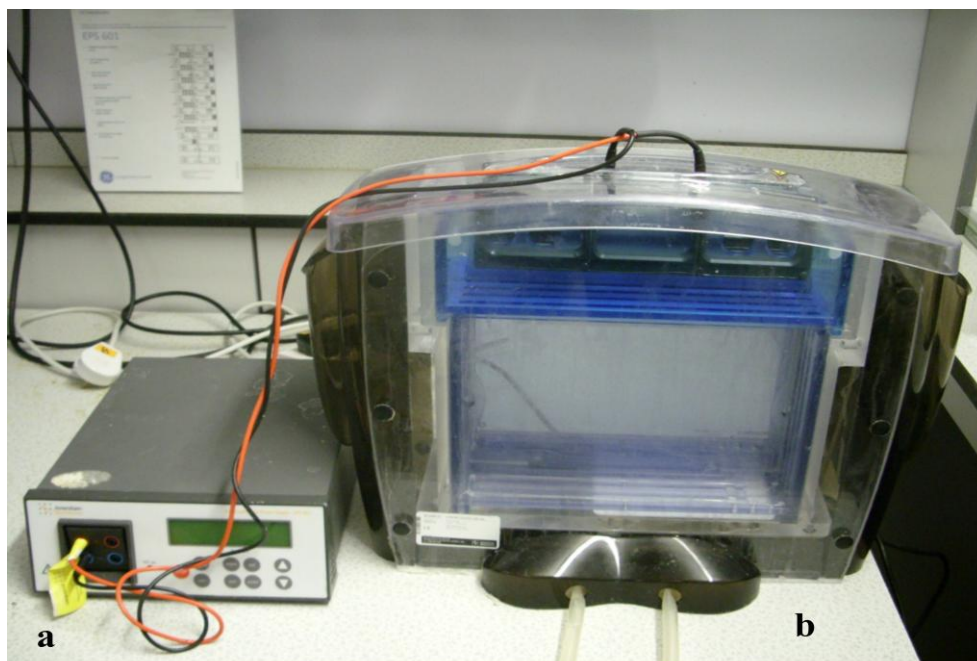


Figure 2.4. (a) Electrophoresis Power Supply EPS 601, (b) 2D-gel tank for SDS-PAGE separation.

2.12. PDQuest Spot Analysis

PDQuest-Analysis version 8.0 was used to analyse three replicate gel images from each experiment with each pathogen. The Spot Detection Wizard function in addition to the Gaussian Spot Boundary Detection function were used to identify spot centres and to detect spot boundaries automatically. To identify any missed spots, the Wizard was used manually.

The spot quality was checked manually and the Contour Spot or Freehand Boundary Tool were used as required to correct the spots with a quality rating of 30 or lower. The spot boundaries also were corrected with an artefact regardless of quality rating. The intensity of spot data were normalized within PDQuest by the Total Density In-gel Image method.

2.13. Protein spot picking

Protein spots were picked from the gels using either an Ettan Spot Picker (Amersham Biosciences) or manually using a scalpel. Excised spots were placed into a microtitre plate and stored at -20°C until they were ready for trypsin digestion.

2.14. In-Gel trypsin digestion protocol

Excised spots were digested using either an Ettan Digester (Amersham Biosciences) or manually for matrix-assisted laser desorption/ionisation (MALDI) or quadrupole-time of flight (Q-TOF) analysis. To digest the protein, the spots were cut into small pieces around 1mm x 1mm square using a scalpel and were put in Eppendorf tubes. The gel pieces were washed in 150 µl SDW for 5 min, then were centrifuged at 13,000 g for 3 min. The supernatant was removed and 150 µl of acetonitrile (Fisher Scientific) was added and left for 10-15 min until the gel pieces had shrunk. The acetonitrile was removed after centrifugation and 50 µl of a reduction buffer comprising 10 mM DTT in 0.1 mM ammonium bicarbonate (NH₄ HCO₃) was added and incubated for 30 min at 56°C. The reduction buffer was removed and acetonitrile was added until the gel pieces had shrunk (10-15 min). They were centrifuged as before for 3 min, the supernatant was removed and 50 µl

of alkylation buffer (55 mM iodoacetamide in 0.1 mM NH_4HCO_3) was added. After 30-45 min, the gel pieces were centrifuged immediately for 3 min and the supernatant was discarded. The gel pieces were incubated with 150 μl 0.1 mM NH_4HCO_3 for 15 min, then they were centrifuged as before for 3 min and the supernatant was removed and acetonitrile was added to shrink the gel pieces, before they were dried in a vacuum centrifuge for 5 min. A 150 μl volume of 0.1 mM NH_4HCO_3 and 150 μl of acetonitrile were added to remove any remaining Coomassie stain. The gel pieces were vortexed for 30 s and centrifuged for 3 min, then the supernatant was discarded and acetonitrile was added to shrink the gel pieces before drying in vacuum centrifuge as before. A digestion solution containing 12.5 ng/ μl of trypsin in 50 mM NH_4HCO_3 was prepared, and 20 μl added before incubating at 4°C for 30-45 min. More trypsin solution was added if the liquid had been absorbed completely by the gel pieces. The remaining liquid was removed after a further 30-40 min, and 20 μl of 50 mM NH_4HCO_3 was added. The suspension was incubated at 37°C for 16 h, then 10 μl of a 50% acetonitrile and 50% formic acid solution were added and the mixture sonicated in a sonicating water bath for 10 min. The resulting solution was transferred to new Eppendorf tubes. The sonication step was repeated once or twice, with a further 10-20 μl of the acetonitrile, and formic acid solution added. The gel pieces were discarded and the final peptide solution was stored at -20°C for subsequent mass spectrometry analysis.

2.15. Mass spectrometry

Protein spots were manually cut from the gels and subsequently digested overnight using 20 μl trypsin (20 ng μl^{-1} ; Promega Sequencing Grade Porcine Modified) in 20

mM ammonium bicarbonate (Sigma) at room temperature. The next day the peptides were extracted in 50% acetonitrile/0.1% trifluoroacetic acid into a clean microtitre plate and transferred to an Ettan Spotter (Amersham Biosciences). The peptides were mixed with matrix (10 mg ml⁻¹ α -cyano-4-hydroxycinnamic acid in 50:50 v/v methanol/acetonitrile) for spotting onto Micromass target plates for analysis in a MALDI-TOF mass spectrometer (Waters-Micromass, UK). Analysis of peptides was performed by use of a nitrogen UV laser (337 nm). MS data were acquired in the MALDI reflector positive ion mode in the mass range 800–3,500 Da. Identification of proteins from the mass fingerprints generated was performed using Proteinlynx Global Server software (V2.0.5, Waters-Micromass, UK) which searched the Swissprot and National Centre for Biotechnology Information (NCBI, Bethesda, USA) databases. The search parameters used included a peptide mass tolerance of 100 ppm, estimated calibration error of +0.025 Da, fixed carbamidomethylation of cysteine, variable oxidation of methionine and one missed cleavage per peptide.

A Q-TOF Micro mass spectrometer (Waters-Micromass, UK) coupled to a LC Packings capillary liquid chromatography system was used to acquire nanoelectrospray ionization tandem mass spectra. 15 μ l aliquots of peptide solutions prepared as described above were injected using an auxiliary solvent flow of 30 μ l min⁻¹ and desalted on a C₁₈ PepMap Nano-Precolumn (5 \times 0.3 mm internal diam (i.d.), 5 μ m particle size; Dionex, Amsterdam, The Netherlands) for 4 min. Peptides were eluted and separated by use of a C₁₈ Pep Map100 nano column (15 cm \times 75 μ m i.d., 3 μ m particle size) with a gradient flow of 200 nl min⁻¹ and solvent system of: auxiliary solvent, 0.1% HCOOH; solvent A, 5% v/v CH₃CN/95% v/v 0.1% v/v aqueous HCOOH; solvent B, 80% v/v CH₃CN/20% v/v 0.1% v/v aqueous

HCOOH. The solvent gradient was 4 min at 5% aqueous solvent B, 5% to 55% B over 40 min, 55% to 80% B over 1 min, maintained at 80% B for 5 min, then reduced to 5% B in 0.1 min and the column washed with solvent A for 9.9 min before the next sample injection. A short length of 75 μm i.d. capillary was used to connect the column to the nanosprayer of the Z-spray ion source. The following voltages were used: 3,500 V for the capillary, 45 V for the sample cone and 2.5 V for the extraction cone. MS spectra were acquired throughout the chromatographic run, while MS/MS spectra were acquired in data-dependent mode on the most abundant ions having charge states of 2+, 3+ and 4+ between m/z 400–2,000. The collision cell was pressurised with 1.38 bar ultra-pure argon (99.999%, BOC) and collision voltages depended on the m/z and charge states of the parent ions. Daily calibration of the mass spectrometer was performed using MS/MS fragment ions from [Glu¹]-fibrinopeptide B (Sigma). The Swissprot and NCBI databases were searched following the submission of processed data to the ProteinLynx Global Server (V2.0.5) and also to MASCOT (Matrix Science). Search criteria were: peptide tolerance of 100 ppm; fragment tolerance of 0.1 Da; fixed carbamidomethylation of cysteine; variable oxidation of methionine modifications and two missed cleavages per peptide.

2.16. Experimental design for samples used for 2D-gel electrophoresis

Pea seeds were sown in compost or autoclaved soil, then grown in the same conditions at 20°C in a cycle of 16 h light and 8 h dark for different periods of time before they were inoculated with different pathogens (*P. viciae*; *F. solani* and *M. hapla*). Proteins were extracted from pea leaves and roots using different protocols before the proteins were separated by using 1D- and 2D-gel electrophoresis as

discussed in detail above. Control plants were subjected to the same treatment as each of the pathogens. The flow chart in Figure 2.5 summarises these procedures.

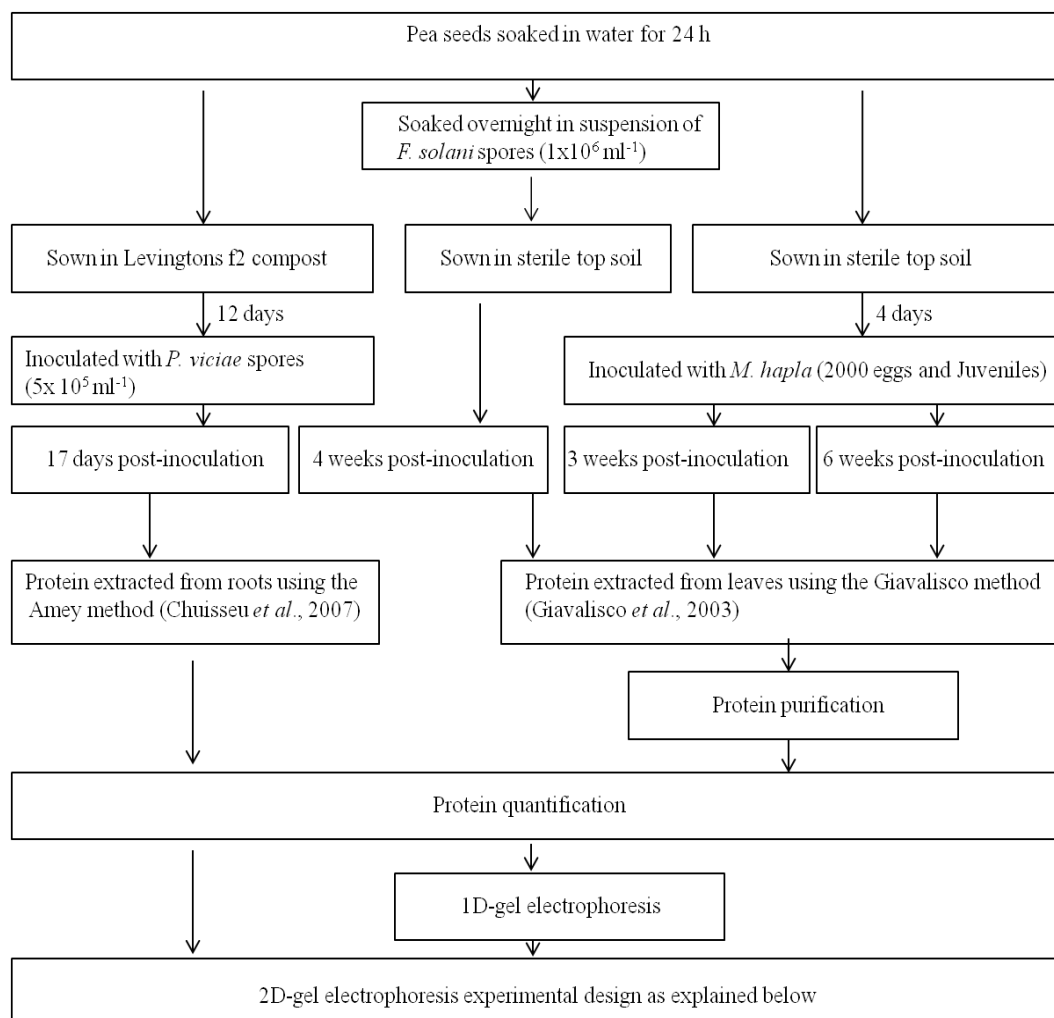


Figure 2.5. Flow chart summarising the inoculation and protein extraction protocol used to prepare samples for 1D- and 2D-gel electrophoresis.

2D gel experiments were designed as shown in Fig. 2.6. Each experiment comprised three biological replicate samples of plant tissue, and each biological replicate comprised 3 gels of proteins from healthy and three gels of protein from infected samples. The 2D gels were analysed using PDQuest software to select proteins for picking from the gels and digesting using trypsin as described in section

2.14. The peptide extracts were identified by using MALDI-TOF and Q-TOF analysis as described in section 2.15.

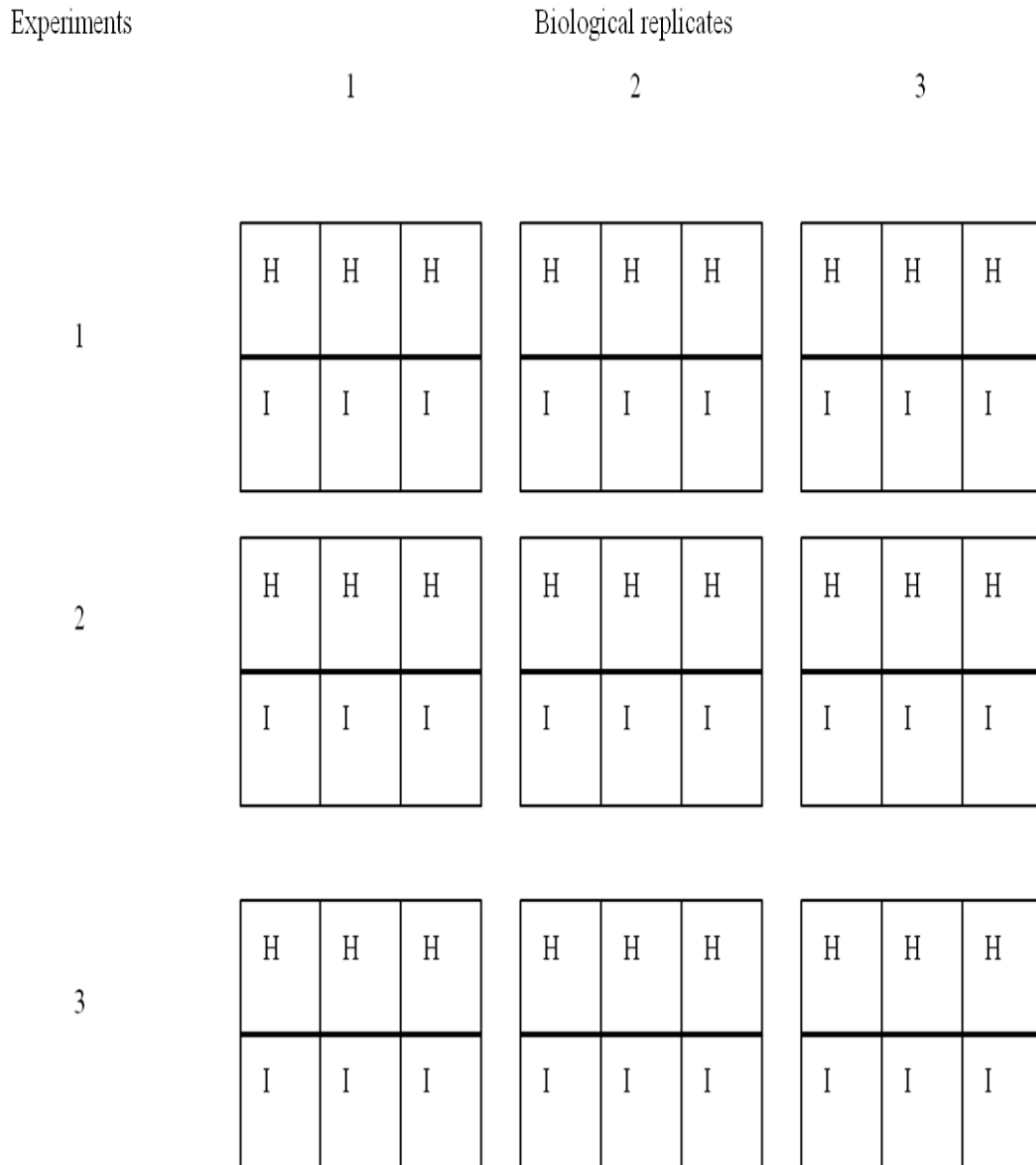


Figure 2.6. Experimental design for 2D gels of proteins from pea plants inoculated with different pathogens. H = gels of healthy and I = gels of infected (*P. viciae*, *F. solani* or *M. hapla*) pea plants.

2.17. Estimation of leaf area and plant biomass

2.17.1. Leaf area

Several methods of leaf area measurements have been developed; one of these methods which has been used in this study is image J which is Java-based image processing program developed at the national institute of health, USA (NIH) by Wayne Rasband (<http://rsb.info.nih.gov/ij/>).

2.17.2. Plant biomass

Shoots and root systems of healthy and infected pea plants were weighed fresh and after they were dried overnight in an oven at 100°C.

2.18. Visualization of leaves using normal and infrared camera

Normal and infrared camera type JAI AD-080CL 2CCD multiple spectral cameras have been used in this study to differentiate between leaves from healthy and infected plants.

3. Results

3.1. Healthy plants

3.1.1. Protein extraction from pea roots

Protein extraction is a crucial step in 1D and 2D gel analysis of proteins, and can have a significant impact on both the quantity and quality of detected proteins. Protein extraction from plant tissues is a big challenge because of a low quantity of proteins and the presence of contaminating compounds. Therefore the initial focus was to find the optimal protein extraction method. Six methods of protein extraction were compared and the best method was chosen according to simplicity, and efficiency.

The quality of these methods was compared using 1D and 2D gels as shown below (Figs 3.1 and 3.5), after different procedures had been conducted such as purification, measuring the conductivity of crude extracts and quantification ($\mu\text{g}/\mu\text{l}$) as shown in sections 3.1.1.2, 3.1.1.3 and 3.1.1.4.

3.1.1.1. 1D-gel electrophoresis (SDS PAGE)

The amounts of protein extracted were compared by loading onto 1D gels to compare the number and intensity of bands. Figure 3.1 shows a representative gel comparing the six methods assessed. The Brigham method (Brigham *et al.*, 1995) was the least efficient, followed by the SDS and SDS-acetone extraction methods (Shultz *et al.*, 2005), BPP method (Wang *et al.*, 2007) and then the Amey method (Chuisseu *et al.*, 2007), whilst the TCA-acetone method (Natarajan *et al.*, 2005) was the best as it gave the largest protein yield. Therefore the TCA-acetone and Amey methods for protein extraction were explored in subsequent experiments.

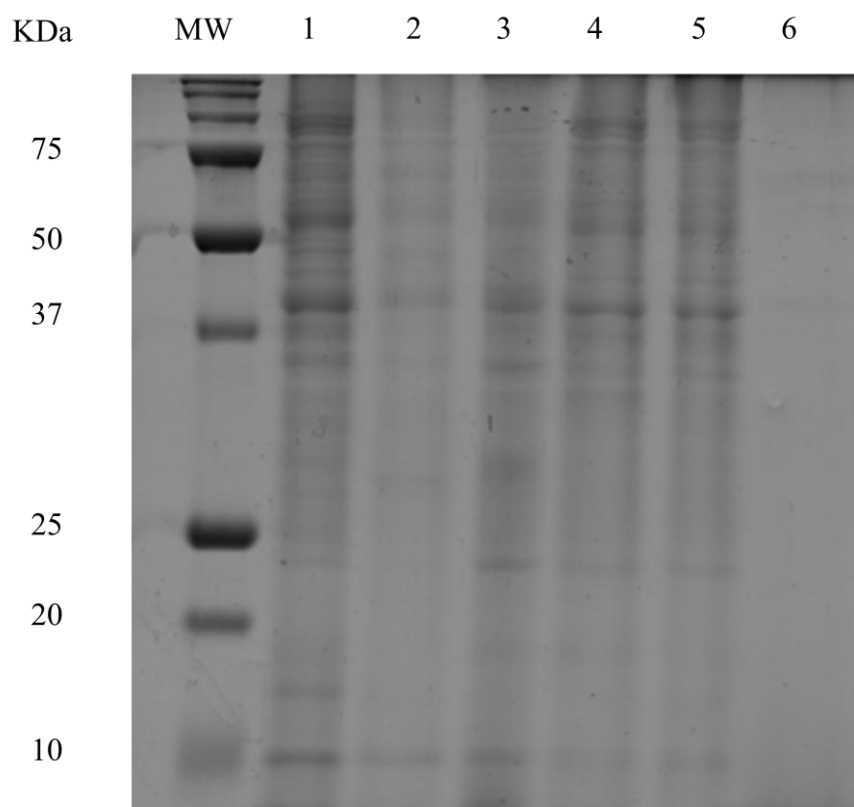


Figure 3.1. 1D gel of proteins extracted from pea roots by six methods: 1, TCA-acetone; 2, SDS; 3, SDS-acetone; 4, Amey; 5 BPP; 6, Brigham. All lanes were loaded with 15 μ l protein extract (see Materials and Methods for details); MW = molecular weight markers, KDa, (Bio-Rad, UK).

The effect of different quantities of protein solutions added to 1D gels was compared (Fig. 3.2). The results showed no difference in the number and intensity of protein bands when 15 and 20 μ l volumes were loaded, compared to faint bands with 5 and 10 μ l. Therefore 15 μ l was used in all future work.

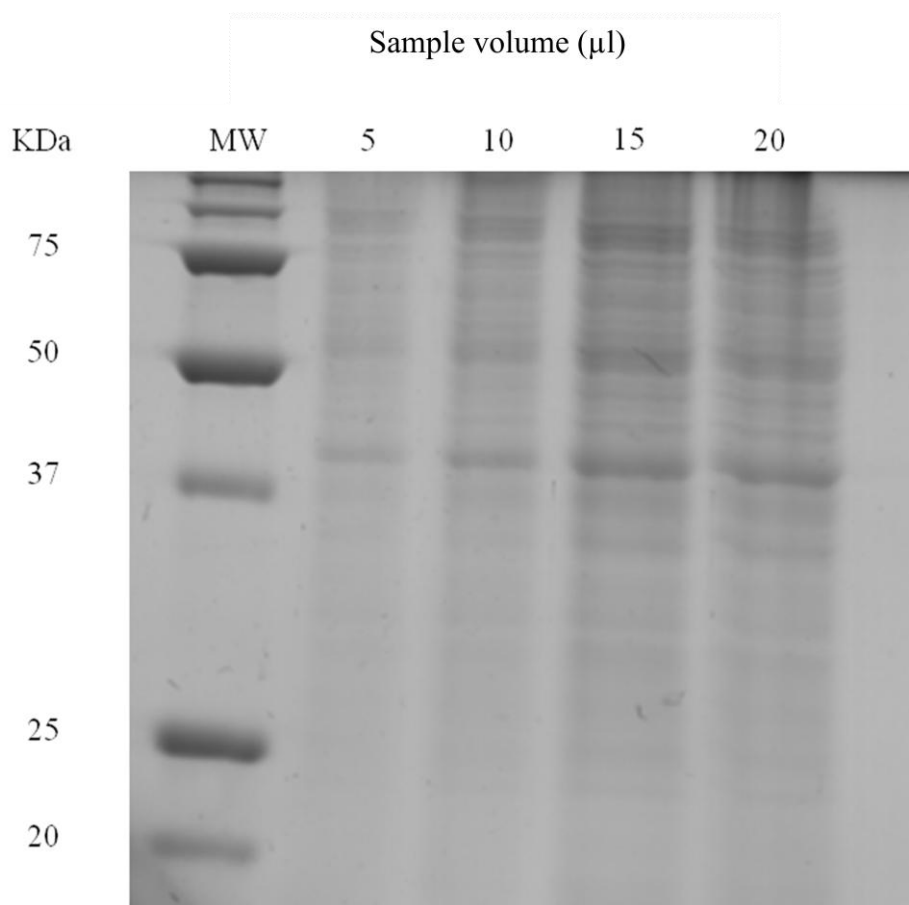


Figure 3.2. A representative 1D gel shows 4 different amounts of proteins (5, 10, 15, 20 μ l), extracted using the TCA-acetone method. MW = molecular weight markers, KDa.

Comparison of data for protein separation by 1D- (this section) and 2D-gel electrophoresis (section 3.1.1.5) showed the Amey method (Chuisseu *et al.*, 2007) as being most suitable. Therefore the possible interference of salts was explored by measuring conductivity and by comparing desalting methods on extracts prepared using the Amey method.

3.1.1.2. Purification of protein extracts

Two methods of purification were compared for their efficiency in cleaning protein extracts. They were needed because the proteins extracted from pea roots is a crude solution that might contain some contaminants and interfering compounds that would affect the running of gels. Two methods were evaluated to overcome these problems, and the best method chosen for further work.

The results showed that many proteins were lost when using 2D Clean-up Kit (GE Healthcare), compared to no proteins being lost when using the Zeba column (Precise, UK), as shown in Figures 3.3 and 3.4 below.

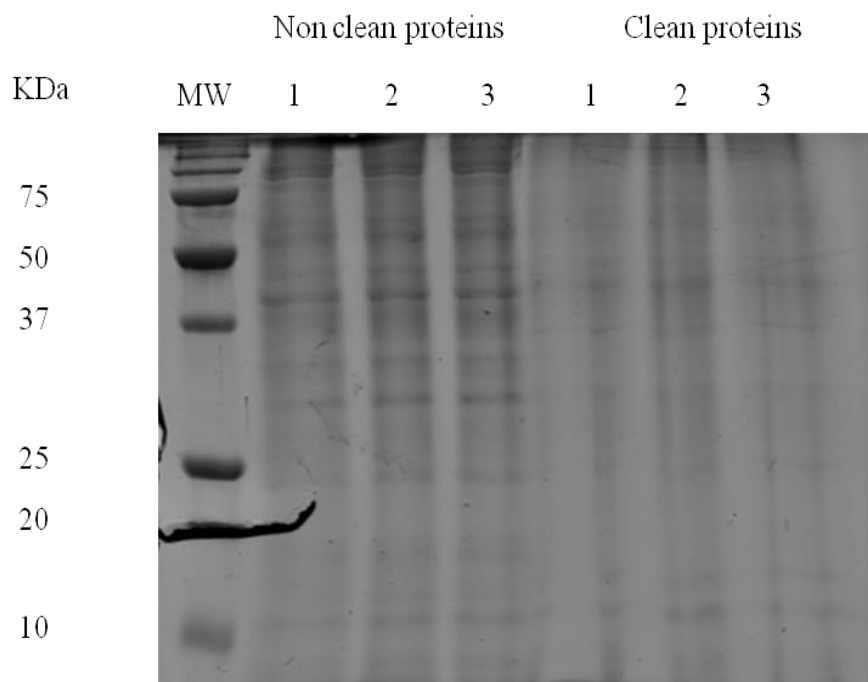


Figure 3.3. 1D gel of proteins extracted from pea roots using the Amey method in 3 biological replicates (1, 2, 3) and either treated using the GE Healthcare 2D Clean-up Kit (clean protein) or untreated (non-clean protein). MW = molecular weight markers, KDa; 15 μ l was loaded in each lane.

In contrast, the Zeba De-salt Spin Columns did not show any loss of proteins as shown in Figure 3.4, but did not give good results in 2D gels compared to the 2D Clean-up Kit (data not shown). However, as the conductivity experiments showed very low salt concentrations in protein solutions extracted using the Amey method, the necessity of protein purification was ruled out.

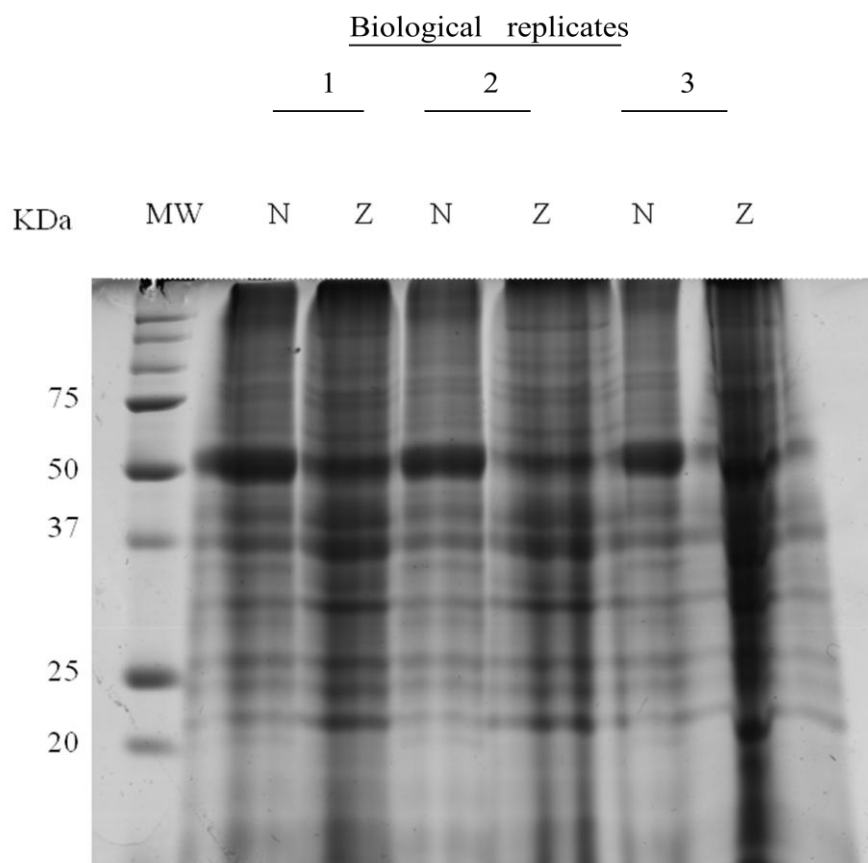


Figure 3.4. 1D gel of proteins extracted from pea roots using the Amey method in 3 biological replicates (1, 2, 3) and either treated using the Zeba De-salt Spin Column (Z) or untreated (N). MW = molecular weight markers, KDa; 15 μ l was loaded in each lane.

3.1.1.3. Conductivity of extracted protein solutions

Conductivity is defined as the ability of a material to conduct electric current, and the basic unit of measuring conductivity is known as a Siemens (S). The

conductivity of a solution depends on the concentration of dissolved salts and other chemicals that release ions into the solution. The conductivity experiments showed the amount of salt in protein solutions from pea roots extracted using the Amey method, compared with deionised water and a range of NaCl concentrations (Table 3.1).

Table 3.1. Conductivity of protein solutions extracted from pea roots using the Amey method (Chuisseu *et al.*, 2007), compared to conductivity of deionised water and NaCl solutions in three replicates of each.

Replicate number	Conductivity (mS/cm)				
	Protein solution	Deionised water	NaCl, 50 mM	NaCl, 100 mM	NaCl, 150 mM
1	6.14	1.3	7.28	12.60	18.55
2	6.14	1.2	7.20	12.60	18.50
3	6.15	1.4	7.25	12.63	18.54
Mean	6.14	1.3	7.24	12.61	18.53

The conductivity of deionised water at 1.3 mS/cm is higher than a published value of 0.55 mS/cm (Pashley *et al.*, 2005). The data showed that whilst the conductivity of the protein solution is higher than deionised water, it is also significantly lower than the conductivity of 50 mM NaCl. Therefore the salt concentration of proteins extracted by the Amey method is lower than concentrations likely to cause problems in 2D-gel electrophoresis, which are 50 mM and above (Gorg *et al.*, 2004; Anon., 2008). This means that there was no need to remove salts from the protein extracts prepared by the Amey method when running 2D gels to separate pea root proteins.

3.1.1.4. Protein quantification

The Bradford assay (Bradford, 1976) was used to quantify proteins extracted using the TCA-acetone (Natarajan *et al.*, 2005) and the Amey (Chuisseu *et al.*, 2007) methods to confirm the difference in the efficiency between these two methods as indicated by 1D-gel electrophoresis experiments (Fig. 3.1).

The amount of protein extracted using the TCA-acetone method (1.16 $\mu\text{g}/\mu\text{l}$) was significantly more ($p < 0.05$) than the amount of protein extracted using the Amey method (0.60 $\mu\text{g}/\mu\text{l}$; Table 3.2).

Table 3.2. Concentration of protein extracted from roots of healthy pea plants using the Amey and TCA-acetone methods. Data from 3 experiments, each of 3 biological replicates.

Method	Replicate	Protein concentration ($\mu\text{g}/\mu\text{l}$)			
		Experiment 1	Experiment 2	Experiment 3	Mean
Amey	1	0.62	0.66	0.69	0.60
	2	0.63	0.52	0.68	
	3	0.61	0.34	0.60	
	Mean	0.62	0.51	0.66	
TCA	1	1.22	1.08	0.85	1.16
	2	1.14	1.41	1.51	
	3	1.34	0.55	1.38	
	Mean	1.23	1.01	1.25	

3.1.1.5. 2D-gel electrophoresis

Protein samples extracted using the TCA-acetone (Natarajan *et al.*, 2005) and the Amey (Chuisseu *et al.*, 2007) methods were stored at -80°C and then treated with different procedures before resolving in 2D gels. These procedures included solubilisation, denaturation and reduction, as described in the Materials and Methods, to complete separation of the proteins.

Whilst 1D-gel electrophoresis and protein quantification indicated that extraction of proteins by the TCA-acetone method was more effective, 2D gels showed that the Amey method gave many spots (Fig. 3.5) whilst the TCA-acetone method gave no spots (data not shown).

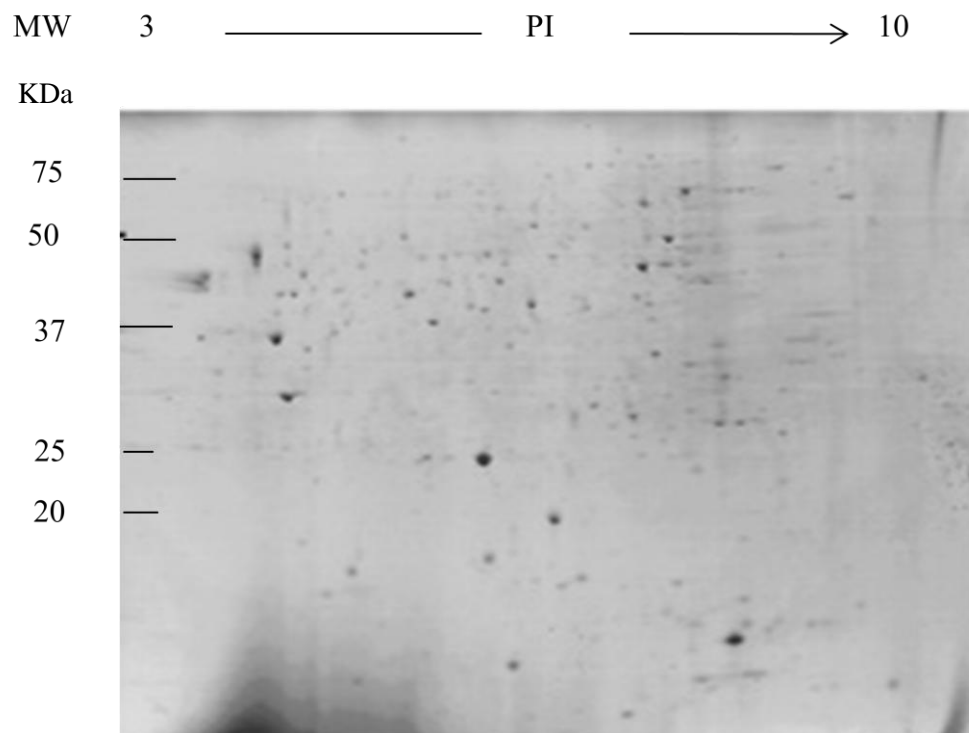


Figure 3.5. 2D gel of proteins extracted from roots of healthy pea plants using the Amey method. PI = pH gradient from 3-10; MW = molecular weight markers, KDa.

3.1.2. Protein extraction from pea leaves

Two methods were evaluated for extracting protein from pea leaves, the Giavalisco *et al.* (2003) and the Amey (Chuisseu *et al.*, 2007) methods. Proteins extracted using these methods were loaded in 1D and 2D gels as shown in (Figs 3.6 and 3.7), after they were subjected to the purification and quantification procedures in sections 3.1.2.1 and 3.1.2.2.

3.1.2.1. Protein purification

Pea leaves contain many contaminants that affect protein separation using 1D- and 2D-gel electrophoresis. Preliminary quantification experiments showed that inaccurate data were obtained when crude protein (before cleaning with the 2D Clean-up Kit) was used in the protein assays. Therefore all protein samples were purified to eliminate the contaminants prior to quantification and running the gels. As reported in section 3.1.1.2, the 2D Clean-up Kit was used to purify the protein samples because it gave better results with 2D gels compared to Zeba De-salt Spin Column. Therefore the 2D Clean-up Kit was used in all future experiments, and no additional purification methods were required.

3.1.2.2. Protein quantification

The two methods of protein extraction were compared by quantifying the extracted protein cleaned using the 2D Clean-up Kit, and the results (Table 3.3) showed significantly greater ($p < 0.05$) amounts of protein extracted using the Giavalisco method ($0.97 \mu\text{g}/\mu\text{l}$) compared to the Amey method ($0.42 \mu\text{g}/\mu\text{l}$).

Table 3.3. Concentration of protein extracted from leaves of healthy pea plants using the Amey and the Giavalisco methods. Data from 3 experiments, each of 3 biological replicates.

Extraction method	Replicate	Protein concentration $\mu\text{g}/\mu\text{l}$			
		Experiment 1	Experiment 2	Experiment 3	Mean
Amey	1	0.34	0.45	0.21	
	2	0.39	0.41	0.56	
	3	0.37	0.60	0.42	
	Mean	0.37	0.49	0.40	0.42
Giavalisco	1	1.44	0.54	0.98	
	2	1.57	0.76	0.89	
	3	1.41	0.66	0.46	
	Mean	1.47	0.65	0.78	0.97

3.1.2.3. 1D-gel electrophoresis (SDS PAGE)

Three biological replicates of proteins extracted from leaves of healthy pea plants using the Amey and Giavalisco methods were compared by 1D-gel electrophoresis. The results showed that the Amey method gave more dense bands of proteins compared to the Giavalisco method (Fig. 3.6).

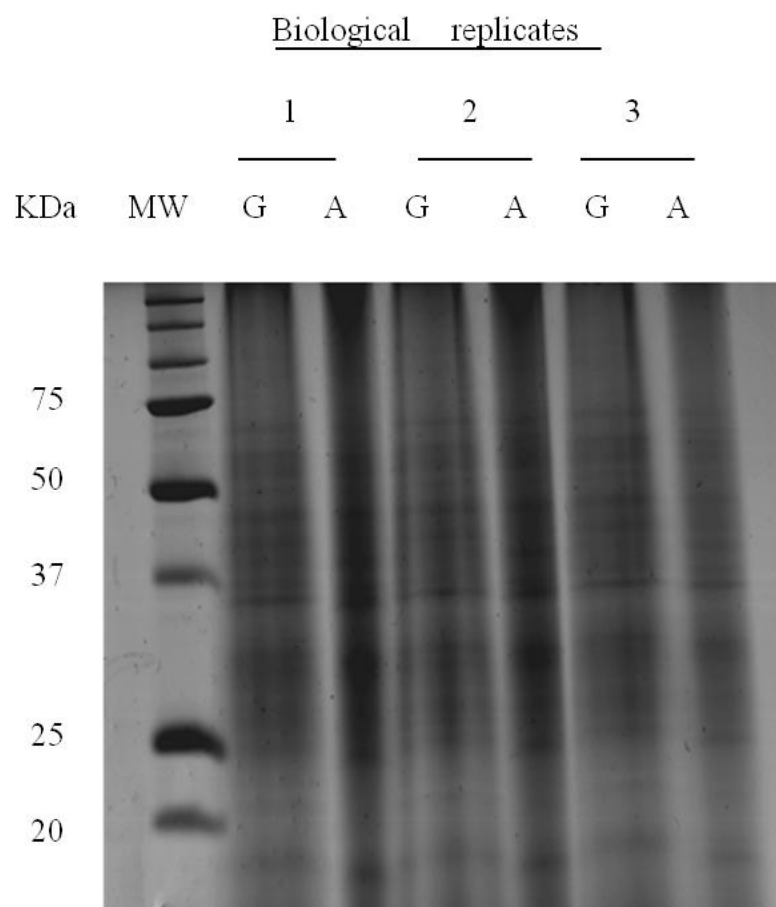
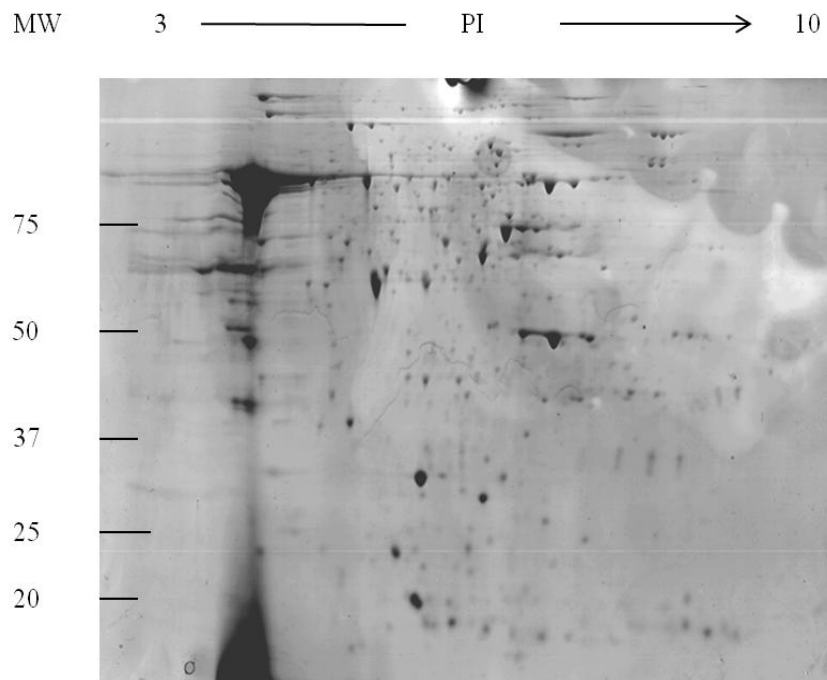


Figure 3.6. 1D gel of protein extracts from pea leaves of healthy plants using the Amey (A) and Giavalisco (G) methods, from 3 biological replicate samples. MW = molecular weight marker, KDa; 15 μ l was loaded in each lane.

3.1.2.4. 2D-gel electrophoresis

Protein samples extracted from leaves of healthy pea plants using the Amey and Giavalisco methods were compared by 2D-gel electrophoresis. The results showed that the Amey method gave more dense protein spots compared to the Giavalisco method (Fig. 3.7). These results contradicted the protein quantification data (see Table 3.3), which showed that the Giavalisco method yielded more protein than the Amey method. Based on the quantification results, however, the Giavalisco method was used in all future experiments.

a. Amey method



b. Giavalisco

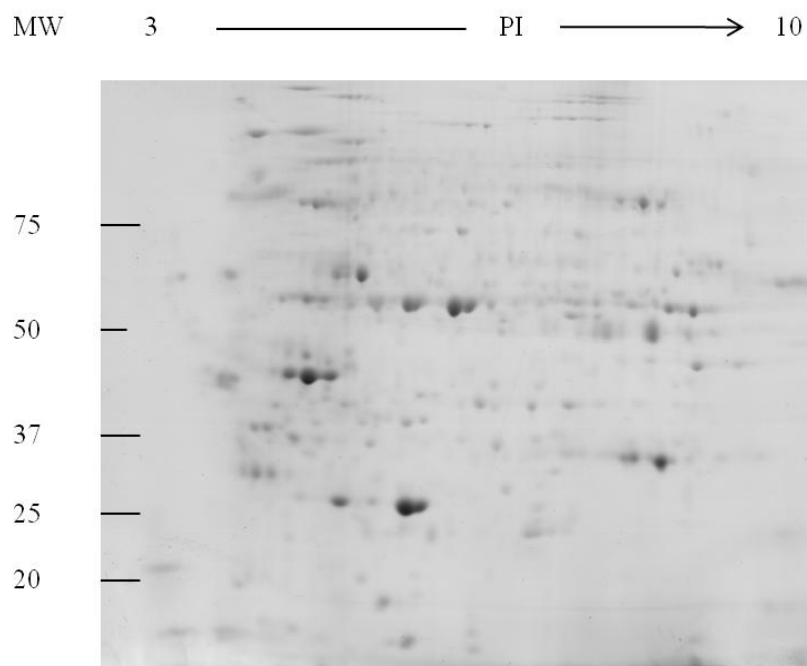


Figure 3.7. 2D gels of proteins extracted from leaves of healthy pea plants using the (a) Amey method and (b) Giavalisco method. PI = pH gradient from 3-10; MW = molecular weight markers, KDa.

3.1.3. Effect of plant age on the amount of protein in pea leaves

Proteins were extracted from leaves of healthy pea plants using the Giavalisco method (Giavalisco *et al.*, 2003) at four and six weeks after sowing seeds. The results showed that the concentration of proteins decreased with plant age (Table 3.4). The quantification results were confirmed by separating the extracted proteins by 2D-gel electrophoresis which showed a clear difference in the number of spots and pattern as shown in Fig. 3.8. The average number of spots was approximately 247 at four weeks and 120 at six weeks.

Table 3.4. Concentration of proteins extracted from leaves of healthy pea plants at 4 and 6 weeks old using the Giavalisco method. Data from 3 experiments, each with 3 biological replicates.

	Replicate	Protein concentration ($\mu\text{g}/\mu\text{l}$)			
		Experiment 1	Experiment 2	Experiment 3	Mean
4 weeks old	1	0.55	0.54	0.52	
	2	0.72	0.57	0.61	
	3	0.62	0.77	0.73	
	Mean	0.63	0.63	0.62	
6 weeks old	1	0.52	0.48	0.51	
	2	0.37	0.41	0.40	
	3	0.44	0.48	0.57	
	Mean	0.44	0.46	0.49	

1. Four weeks

2. Six weeks

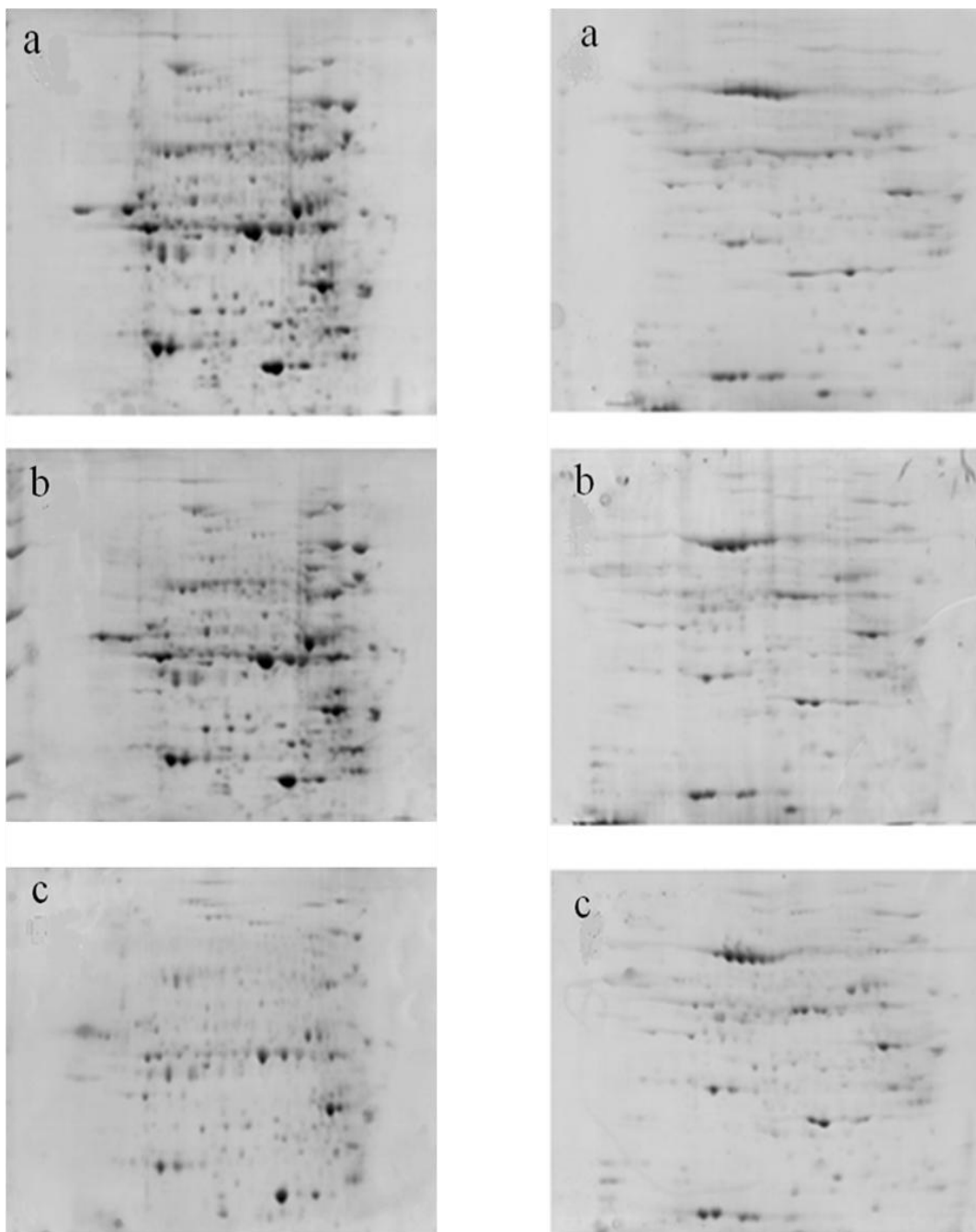


Figure 3.8. Gels of 3 biological replicates of protein extracts from leaves of healthy pea plants at four (1a, b, and c) and six weeks (2a, b, and c) after sowing seeds.

3.2. Infected plants

3.2.1. Downy mildew

3.2.1.1. Pea inoculation

Six cultivars of *Pisum sativum* were inoculated with *P. viciae* f. sp. *pisi* based on the method of Pegg and Mence (1970), and consistently resulted in sporulating infections (Figs 3.9 and 3.10).



Figure 3.9. Sporulating *Peronospora viciae* infection of pea petiole (arrow) and stipule (*). Image from UWE Downy Mildew Research Group.



Figure 3.10. *P. viciae* infected stipule (arrow) and leaf (*) of pea. Source of image as in Figure 3.9.

3.2.1.2. Protein extraction

Two methods of protein extraction were compared to extract protein from roots of *P. viciae* infected pea plants, the Amey method (Chuisseu *et al.*, 2007) and the TCA-acetone extraction method (Natarajan *et al.*, 2005), as for protein extraction from roots of healthy plants. The efficiency of the two methods was compared by quantifying the protein content using the Bradford assay (Bradford, 1976), and by 1D- and 2D-gel electrophoresis.

Protein quantification showed that the amount of protein in roots from *P. viciae* infected plants is less than the amount of protein in roots from non-infected plants. The mean concentration was 0.79 $\mu\text{g}/\mu\text{l}$ in roots from infected plants and 1.13 $\mu\text{g}/\mu\text{l}$ in roots from non-infected plants using the TCA-acetone method, compared to 0.30 $\mu\text{g}/\mu\text{l}$ in infected and 0.59 $\mu\text{g}/\mu\text{l}$ from healthy plants using the Amey method as

shown in Figure 3.11. The amount of protein extracted by the TCA-acetone method was significantly more than the amount of protein extracted using the Amey method, and in healthy compared to infected plants ($n = 9$, $p < 0.05$ for both methods).

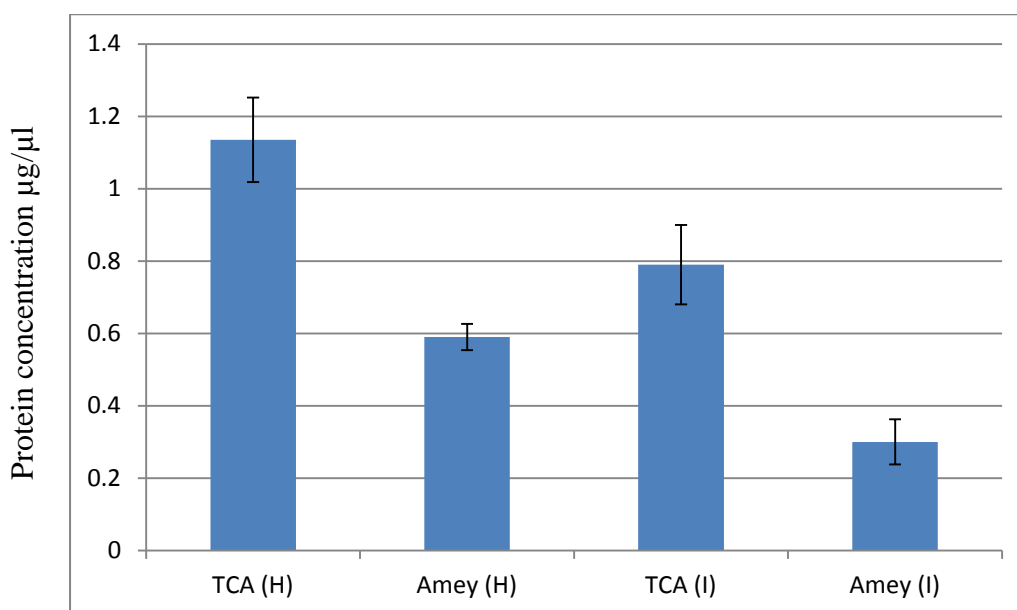


Figure 3.11. Comparison of root protein concentration in extracts from roots of healthy plants (H) and plants infected by *P. viciae* (I), extracted using the TCA-acetone (Natarajan *et al.*, 2005) and the Amey methods (Chuisseu *et al.*, 2007).

3.2.1.3. 1D-gel electrophoresis (SDS PAGE)

The difference in protein concentration between roots of healthy and *P. viciae* infected plants was confirmed when loading equal volumes of protein extract using the TCA-acetone (Natarajan *et al.*, 2005) and Amey (Chuisseu *et al.*, 2007) methods on 1D gels (Figs 3.12 and 3.13). The amount of protein decreased in roots from plants infected by *P. viciae* compared to roots from non-infected (control) (Figs 3.12 and 3.13). Each lane in the representative gels shown was loaded with the

same volume of protein extracts (15 μ l). In each set of replicate extracts, the bands were less intense in protein solutions from roots of infected plants.

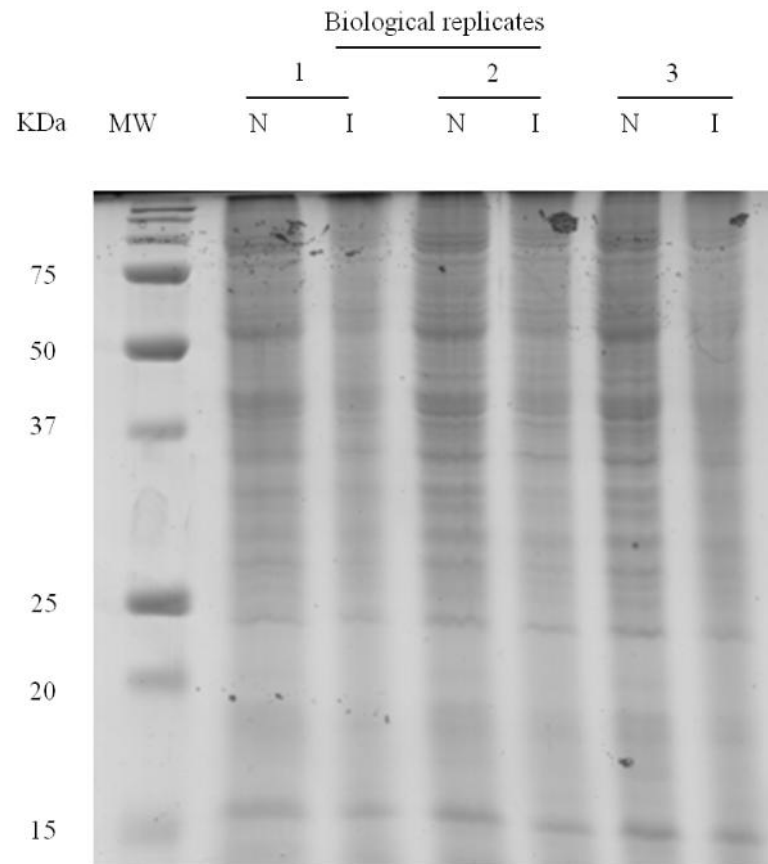


Figure 3.12. 1D gel comparing proteins extracted from roots of *P. viciae* infected (I) and non-infected (N) pea plants using the TCA-acetone method. Data show 3 biological samples, with 15 μ l loaded in each lane. MW = molecular weight markers, KDa.

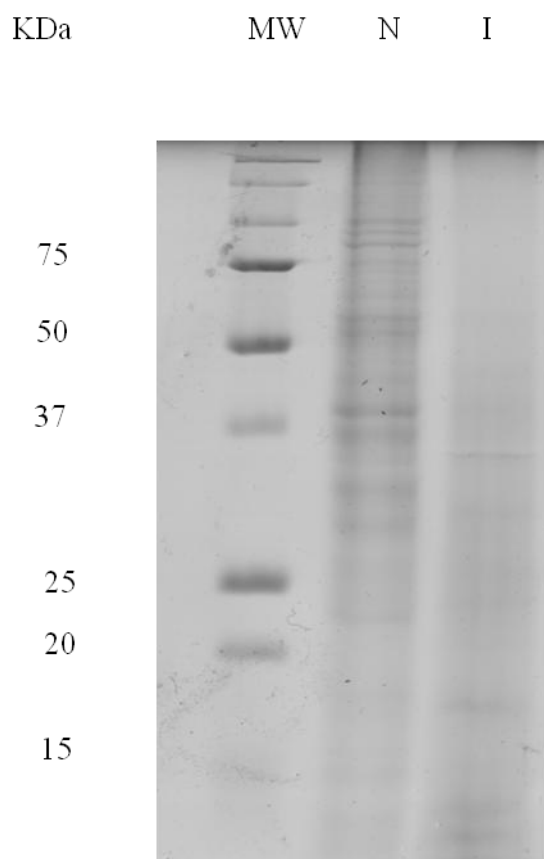


Figure 3.13. 1D gel comparing proteins extracted from roots of *P. viciae* infected (I) and non-infected (N) pea plants using the Amey method, with 15 μ l loaded in each lane. MW = molecular weight markers, KDa.

3.2.1.4. 2D-gel electrophoresis

Equal volumes of protein extract prepared using the Amey method and the TCA-acetone method were used for 2D-gel electrophoresis. Results from protein quantification and 1D-gel electrophoresis previously had shown that the TCA-acetone method was better than the Amey method, based on the amount of protein extracted. In contrast, 2D-gel electrophoresis showed that the Amey method was better than the TCA-acetone method, because it gave a high number of spots (Fig. 3.14) compared to no spots when the protein extracted using the TCA-acetone method was separated using 2D-gel electrophoresis (data not shown).

The three biological replicates shown in Figure 3.14 were subjected to PDQuest analysis and nine spots were chosen because of the difference in their abundance as shown in (Figs 3.15a, b). From these results, the Amey method was chosen to extract proteins from pea roots in all further 2D-gel experiments.

A two-fold threshold was used for indication of significant changes in relative abundance of proteins on 2D gels. No protein spots showed a difference when a four-fold threshold was used. Generally, a 2-fold difference in abundance is accepted as indicating a significant change as long as it is found consistently in all replicate samples (Amey *et al.*, 2008; Rodriguez-Celma *et al.*, 2010). An additional 3D observation of protein spots (Fig. 3.16) showing alteration in relative abundance was performed in order to remove false positives.

Data obtained after gel imaging analysis showed that the nine spots remained different in relative abundance between roots of healthy and *P. viciae* infected pea plants.

1. Healthy

2. Infected

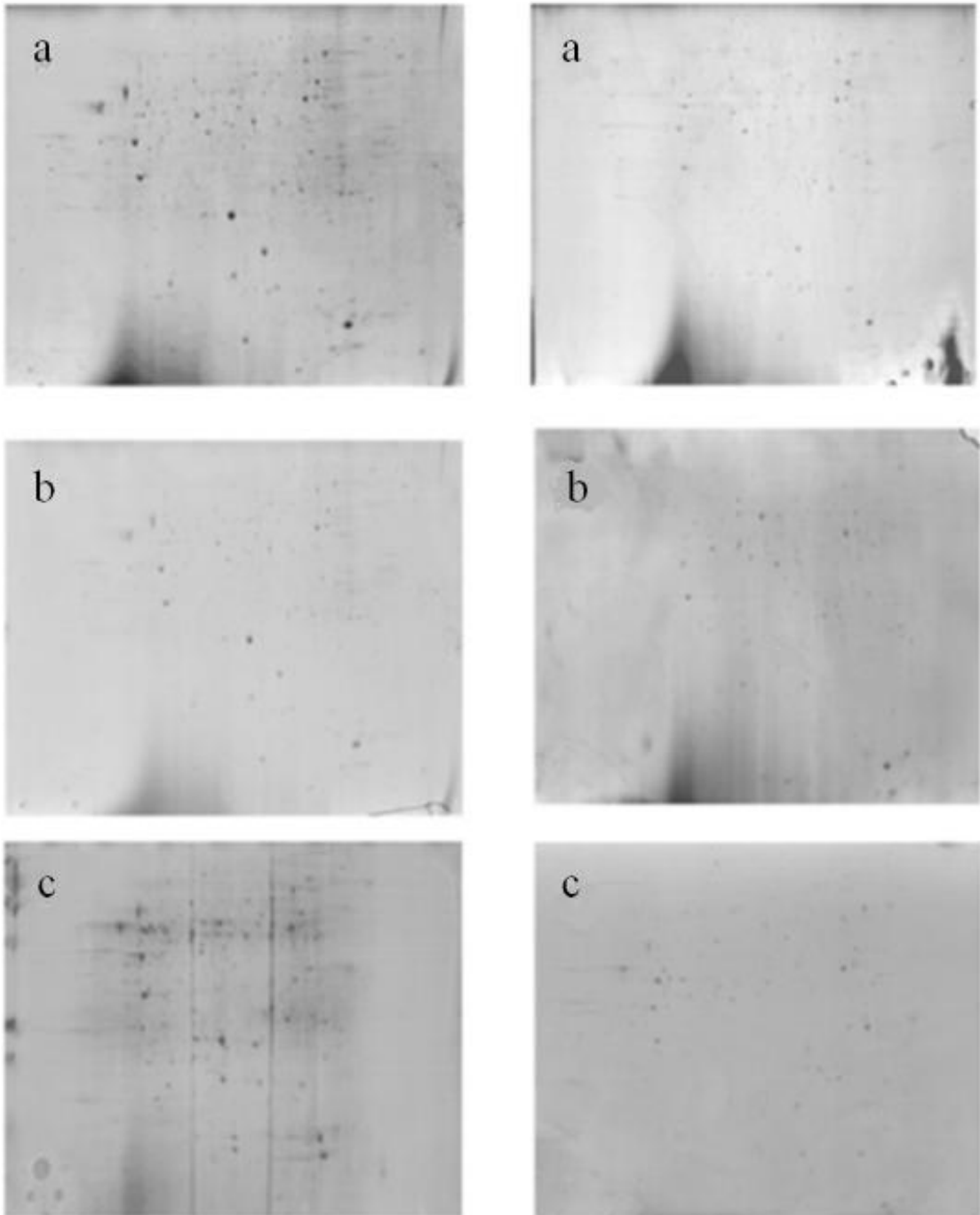


Figure 3. 14. 2D gels of 3 biological replicates of proteins extracted from roots of healthy (1a, b, c) and *P. viciae* infected (2 a, b, c) pea plants.

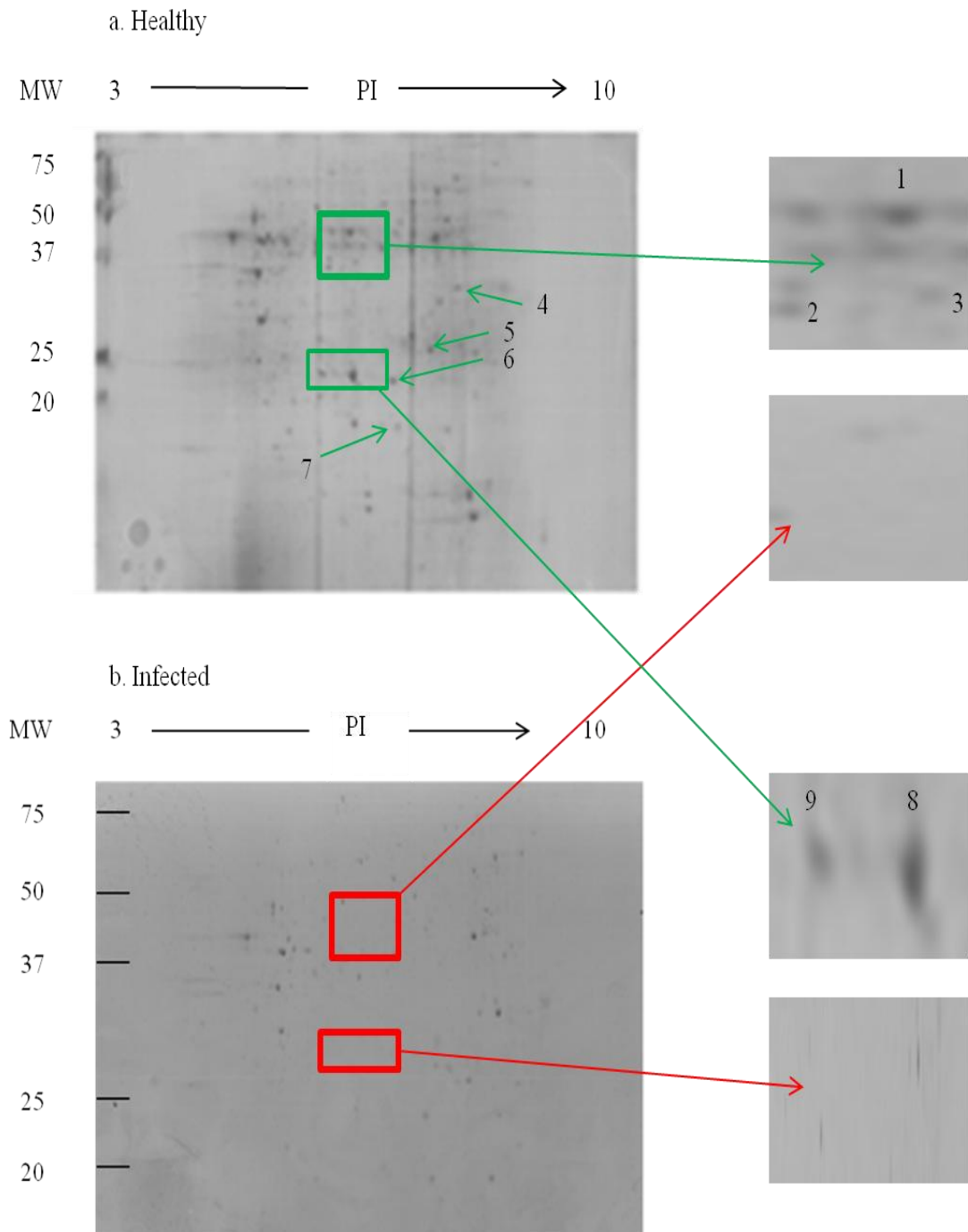


Figure 3.15. Images of 2D gels of proteins extracted from pea roots from (a) healthy plants and (b) plants infected by *P. viciae* (gels 1c and 2c from Figure 3.14) showing in (a) nine protein spots selected for identification using mass spectrometry. Some samples of identified spots are shown as enlarged images. Spot number is the same as in Fig. 3.16 and Table 3.5. PI= pH gradient 3-10; MW = molecular weight (KDa).

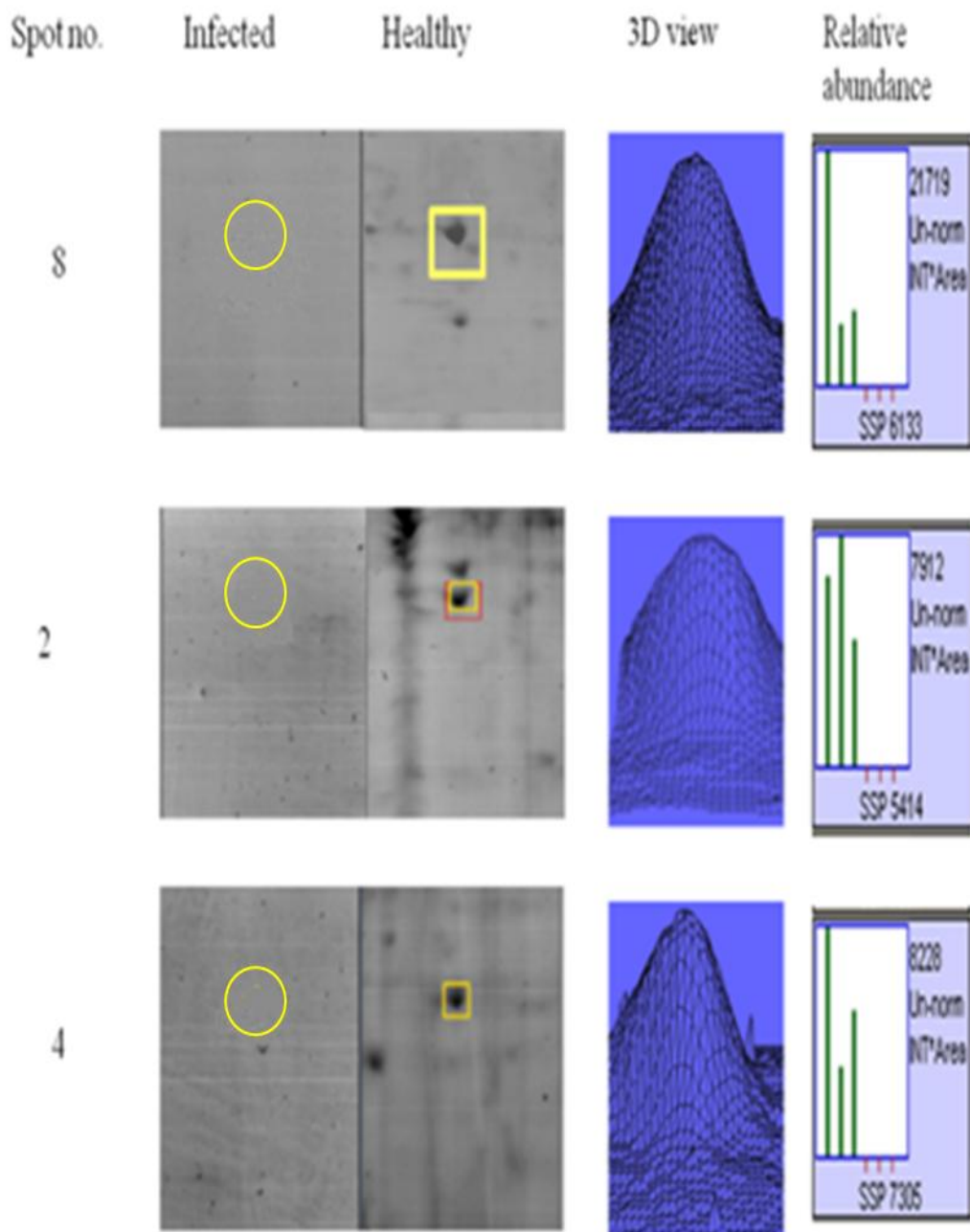


Figure 3.16. Images of three protein spots present in 2D gels of roots of healthy pea plants (square box, numbered as in Fig. 3.15a) and equivalent position on 2D gel of proteins from roots of *P. viciae* infected plants (circles), detected using PDQuest software. For the proteins from roots from healthy plants, the 3D view of the spots and the relative abundance of the protein in each of 3 biological replicates is as shown by PDQuest software.

3.2.1.5. Mass spectrometry and data analysis

On average 229 spots were detected from healthy pea plants compared to 212 spots detected from roots of *P. viciae* pea plants using PDQuest software. The PDQuest software showed that nine spots differed in their abundance in pea roots in response to infection of leaves by *P. viciae* by decreasing at least two fold (Fig. 3.15a). The selection of these spots for MALDI-TOF and ESI-Q-TOF MS/MS was on the basis that their abundance altered significantly and reproducibly on all gel replicates of the different biological samples.

To ensure correct protein identity, these spots were subjected first to robotic digestion followed by MALDI-TOF analysis, but few peptides were identified. The spots were then digested manually and analysed again using MALDI-TOF and two proteins were identified. Protein 1 was matched as glucan endo -1, 3-beta-glucosidase from pea, and shown to have a 3502 Da difference between observed and predicted molecular weights, but a similar iso-electric point (Table 3.5). Protein 3 was identified as alcohol dehydrogenase 1 from pea, with similar predicted and observed molecular weights and iso-electric point. The other spots were subjected to further analysis using Q-TOF analysis. Protein 2 was matched as alcohol dehydrogenase 1 from pea, with a 4870 Da difference between predicted and observed molecular weight values, but similar iso-electric point. Protein 4 was identified as matching isoflavone reductase from *Arabidopsis thaliana* with a slight difference between observed and theoretical molecular weight but a large difference of 2.84 in observed and predicted isoelectric point. Protein 5 was identified as matching malate dehydrogenase from *Beta vulgaris* with a difference of 7893 Da in predicted and observed molecular weight and 2.01 in iso-electric point. Protein 6 was matched as mitochondrial ATP synthase subunit alpha from pea, with a

difference of 1.39 in observed and predicted iso-electric point and a large difference in observed and predicted molecular weight (the observed molecular weight was less than half the predicted value). Protein 7 was identified as matching eukaryotic translation inhibition factor from *A. thaliana*; this protein had an observed molecular weight greater by 13% than the predicted molecular weight and a 2.09 difference in iso-electric points.

Protein 8 represented the third spot identified as alcohol dehydrogenase 1 from pea (see also spots 2 and 3). Whilst it had a similar predicted and observed iso-electric point, its observed molecular weight was only 60% of the predicted value. The final protein from spot 9 was matched as superoxide dismutase from pea. It had very similar predicted and observed molecular weights but a 1.26 difference between isoelectric points.

Table 3.5. Proteins that differed in abundance in pea roots in response to infection of leaves by *P. viciae*, identified using MALDI-TOF (spots no. 1 and 3) and ESI Q-TOF MS/MS (all other spots). Spots numbered 1-9 are from healthy plants, as labelled in gels in Fig. 3.15a, and decreased in abundance by at least two fold following infection.

Spot no.	Protein matching ¹	Protein accession no.	Organism	Observed/predicted mw (KDa)	Observed/predicted PI	Matched peptides	Sequence coverage %	Score ²
1	(1->3)-betaglucanase	Q03467	<i>Pisum sativum</i>	44526/41024	6.70/6.62	5	17.6	9.6
2	Alcohol dehydrogenase 1	P12886	<i>P. sativum</i>	37000/41870	6.20/6.09	21	37	697
3	Alcohol dehydrogenase 1	P12886	<i>P. sativum</i>	41447/41129	6.85/6.45	14	34.7	12.3
4	Isoflavone reductase	P52577	<i>Arabidopsis thaliana</i>	34500/33773	8.50/5.66	4	9	131

Table 3.5 Continued

5	Malate dehydrogenase	Q9SML8	<i>Beta vulgaris</i>	27917/35810	7.90/5.89	10	24	422
6	Mitochondrial ATP synthase subunit alpha	P05493	<i>P. sativum</i>	24250/55296	7.40/6.01	7	14	311
7	Eukaryotic Translation inhibition factor	Q9X191	<i>A. thaliana</i>	19800/17521	7.50/5.41	2	11	88
8	Alcohol dehydrogenase 1	P12886	<i>P. sativum</i>	25000/41870	6.65/6.09	5	12	92
9	Superoxide dismutase	P27084	<i>P. sativum</i>	25583/25863	5.90/7.16	6	30	141

Foot notes

1. (1->3)-beta-glucanase = glucan endo-1, 3-beta-glucosidase

2. Ion score is $-10 \cdot \log(p)$, where p is the probability that the observed match is a random event. Protein scores are derived from ion scores as a non-probabilistic basis for ranking protein hits.

3.2.2. *Fusarium* root rot

3.2.2.1. Pea inoculation

Fusarium solani f. sp. *lisi* was maintained on PDA in plastic Petri dishes before being used for inoculation of pea plants (see Materials and Methods), which resulted in formation of micro- and macro-conidia (Fig. 3.17).

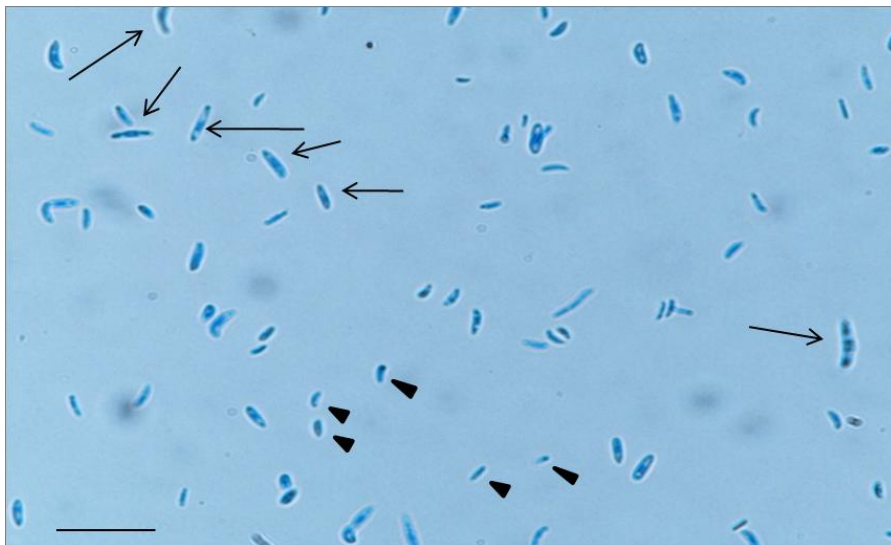


Figure 3.17. *Fusarium solani* f. sp. *lisi* micro- (arrowheads) and macro-conidia (arrows). Scale bar = 50 μ m.

Two methods were used to inoculate plants of *P. sativum* cv. Livioletta with *F. solani*: these were the soil inoculation method of Clarkson (1978), and the seed inoculation method of Kraft and Kaiser (1993) which was modified by Ondrej *et al.* (2008). Both methods gave similar symptoms on the shoot system, with yellowing of leaves and stunting (Figs 3.18 and 3.19). The roots also were stunted compared to roots from healthy plants in both inoculation methods, with a brown colour developing on roots of the infected plants inoculated with seed inoculation method

as shown in Fig. 3.19, but not with the soil inoculation method. The *Rhizobium* nodules were decreased significantly on roots infected by both methods.



Figure. 3.18. Three healthy pea plants on right and three plants infected by *F. solani* on left, four weeks after inoculation by the soil inoculation method, showing stunting of shoots and roots following infection.

The seed inoculation method was more reliable compared to the soil inoculation method, and the symptoms were more severe on the shoot and root system.



Figure 3.19. Healthy pea plant on right and plant infected by *F. solani* on left, four weeks after inoculation by the seed inoculation method. Note the brown colour (arrowed) developing on the roots of the inoculated plant.

3.2.2.2. Protein extraction and purification

The method of Giavalisco *et al.* (2003), referred to here as the Giavalisco method, was used to extract protein from leaves from *F. solani* infected and healthy pea plants. As mentioned earlier, plant tissues contain several contaminants that affect protein separation and quantification, so the crude protein extract solution was purified using the 2D Clean-up Kit (see Materials and Methods) to eliminate contaminants.

3.2.2.3. Protein quantification

Results of protein quantification using the Bradford assay (Bradford, 1976) showed a sufficient yield for separation using the 1D and 2D gels. The concentration of protein in extracts of leaves from healthy pea plants was 0.63 $\mu\text{g}/\mu\text{l}$ (mean of three biological samples) which was greater ($n = 9$, $p < 0.05$) than the concentration of 0.37 $\mu\text{g}/\mu\text{l}$ protein extracted from leaves of *F. solani* infected plants (Table 3.6).

Table 3.6. Concentration of protein extracted from leaves of *F. solani* infected and healthy pea plants using the Giavalisco method. Data from 3 experiments, each of 3 biological replicates.

	Replicate	Protein concentration ($\mu\text{g}/\mu\text{l}$)			
		Experiment 1	Experiment 2	Experiment 3	Mean
Healthy	1	0.55	0.54	0.52	0.63
	2	0.72	0.57	0.61	
	3	0.62	0.77	0.73	
	Mean	0.63	0.63	0.62	
<i>Fusarium</i> infected	1	0.40	0.43	0.57	0.37
	2	0.46	0.26	0.44	
	3	0.30	0.38	0.10	
	Mean	0.39	0.36	0.37	

3.2.2.4. 1D-gel electrophoresis (SDS PAGE)

Protein extracted from leaves of healthy and *F. solani* infected pea plants were compared by loading the same volume (15 μ l) of protein extract from three biological replicates of each onto 1D gels. The results showed that the extracts from leaves from healthy plants gave distinct protein bands in all three biological replicates (Fig. 3.20), with bands from extracts from leaves of *F. solani* infected plants being less intense. This confirms the protein assay data (Table 3.6) which showed that the amount of protein in leaves of healthy plants is more than the amount of proteins in leaves of infected plants. Separation of protein extracts using 2D-gel electrophoresis emphasises the difference between the proteome of leaves of healthy and infected plants.

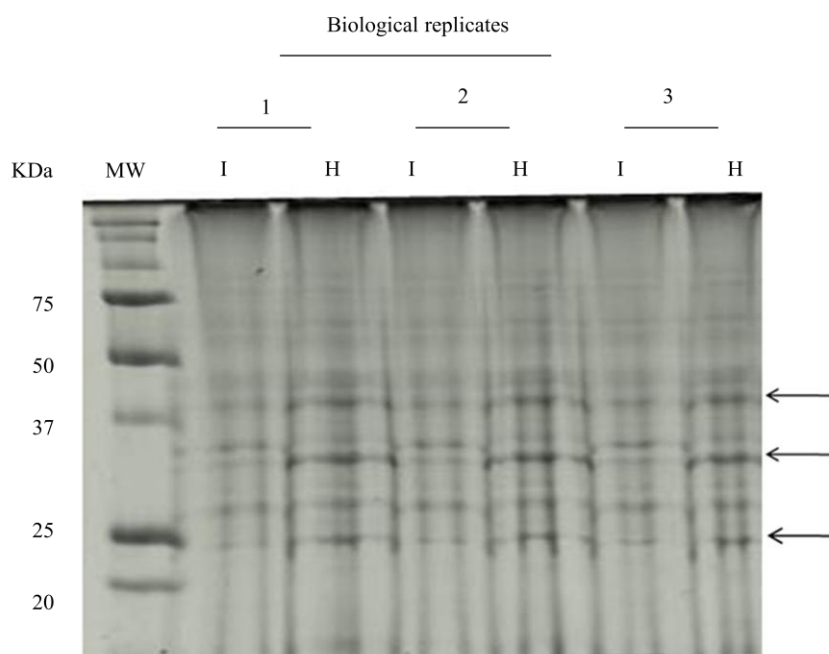


Figure 3.20. A representative 1D gel comparing protein extracted from leaves of healthy (H) and *F. solani* infected (I) pea plants using the Giavalisco method. Arrows indicate protein bands with greater intensity in healthy than infected extracts.

Three biological replicates with 15 µl loaded in each lane. MW = molecular weight markers, KDa.

3.2.2.5. 2D-gel electrophoresis

Protein samples stored at -80°C were thawed and treated with different procedures including solubilisation, denaturation and reduction, before subjecting them to 2D-gel electrophoresis to separate the proteins. Equal amounts of protein extracts from leaves of healthy and *F. solani* infected pea plants from three biological samples were compared on replicate 2D gels.

The results showed similar patterns of proteins between replicate biological samples. Visual observation of the gels indicated a significant difference in the number of protein spots between extracts from leaves of infected and healthy plants (Fig. 3.21), with fewer proteins visible after infection. Gels were subsequently analysed using PDQuest software, followed by excision and digestion of selected spots, which were then subjected to MALDI- and Q-TOF analysis.

Six gels were subjected to PDQuest analysis (Fig. 3.21) to determine the difference in protein abundance between leaves of healthy and *F. solani* infected pea plants. This showed 15 protein spots were different in their abundance following infection (Fig. 3.22), with 12 protein spots decreased in abundance and 3 increased in abundance following infection (Fig. 3.22), when a four-fold threshold was used for the detection of changes in relative abundance. A four-fold threshold was selected as although the protein concentrations and numbers of spots were similar to those in roots of healthy and *P. viciae* infected plants, the scale of difference in abundance was greater. Thus 195 spots were detected from leaves of *F. solani* infected plants and showed a difference in abundance compared to healthy controls of 15 spots with a four-fold threshold but 39 spots with a two-fold threshold. Potential

biomarkers for use in a diagnostic kit is most useful if their abundance changes substantially. Additional 3D observations of protein spots (Fig. 3.23) were performed in order to remove false positives, and confirmed alteration in relative abundance.

1. Healthy

2. Infected

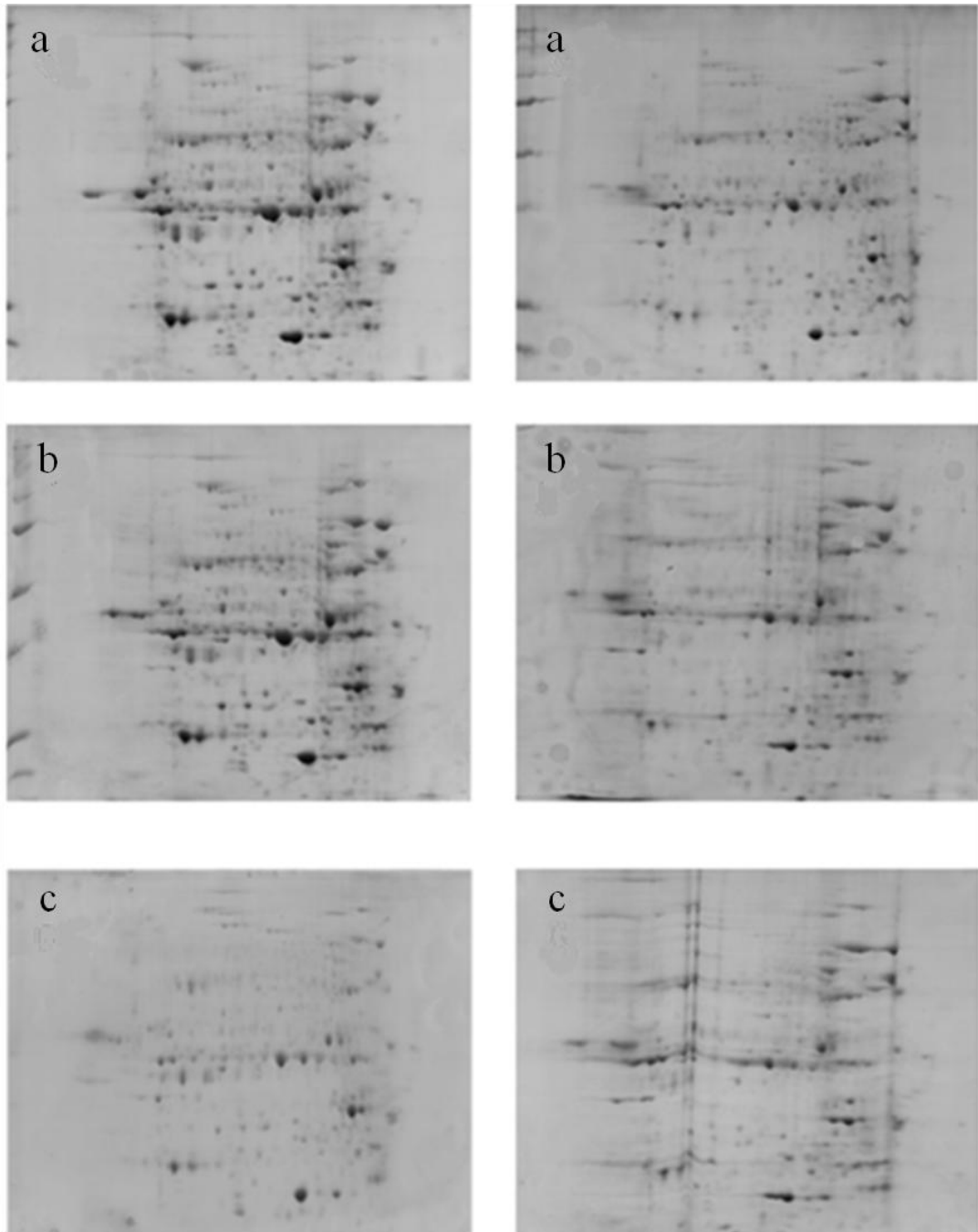


Figure 3.21. Gels of 3 biological replicates of proteins extracted from leaves of healthy (1 a, b, c) and *F. solani* infected (2 a, b, c) pea plants.

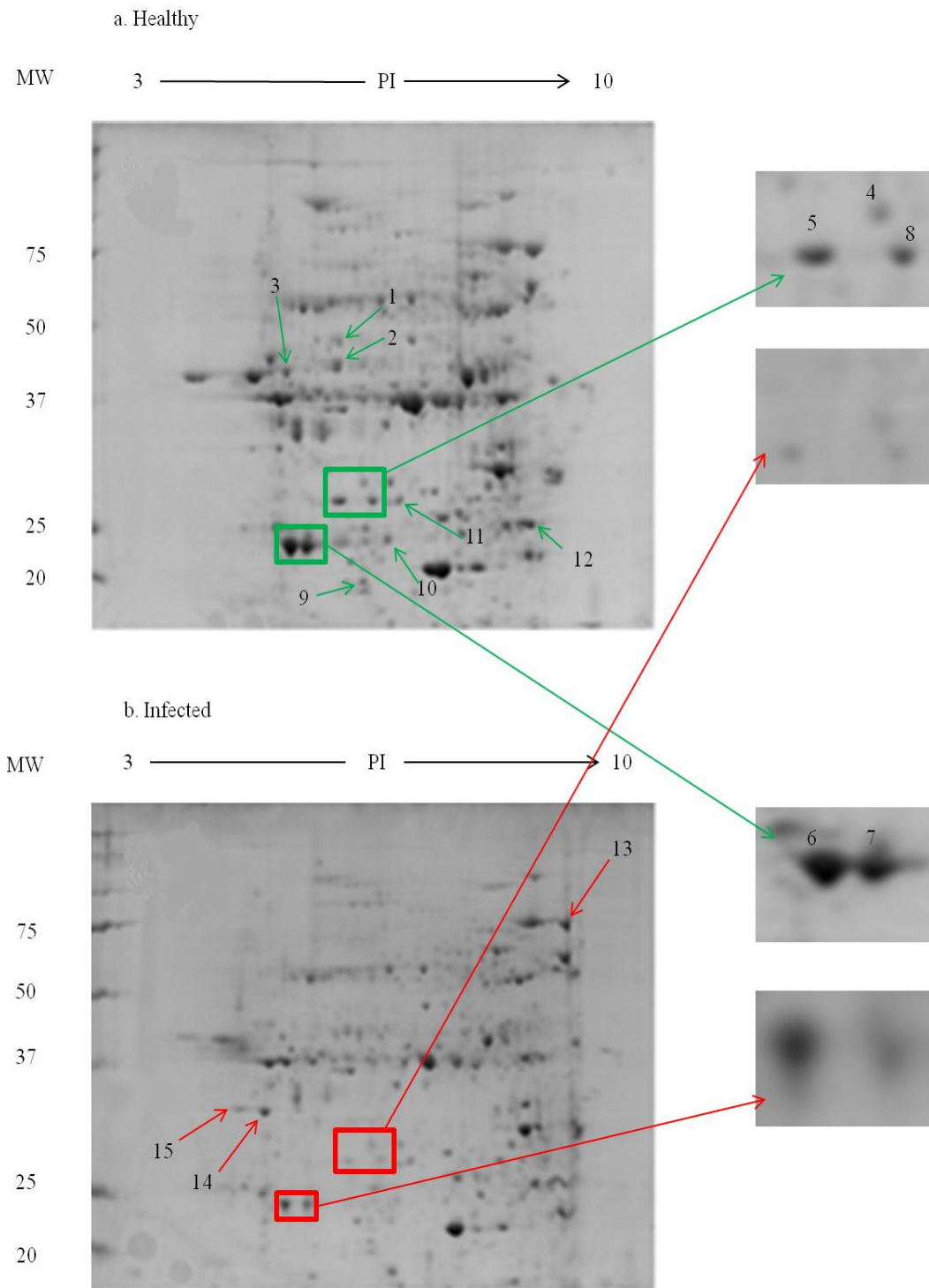


Figure 3. 22. Images of 2D gels of protein extracted from leaves from healthy (a) and *F. solani* infected (b) pea plants (gels 1 and 2a from Figure 3.21) showing 15 spots selected for identification using mass spectrometry. Some samples of identified spots are shown as enlarged images. Spot number is the same as in Fig. 3.23. PI= pH gradient from 3-10; MW = molecular weight (KDa).

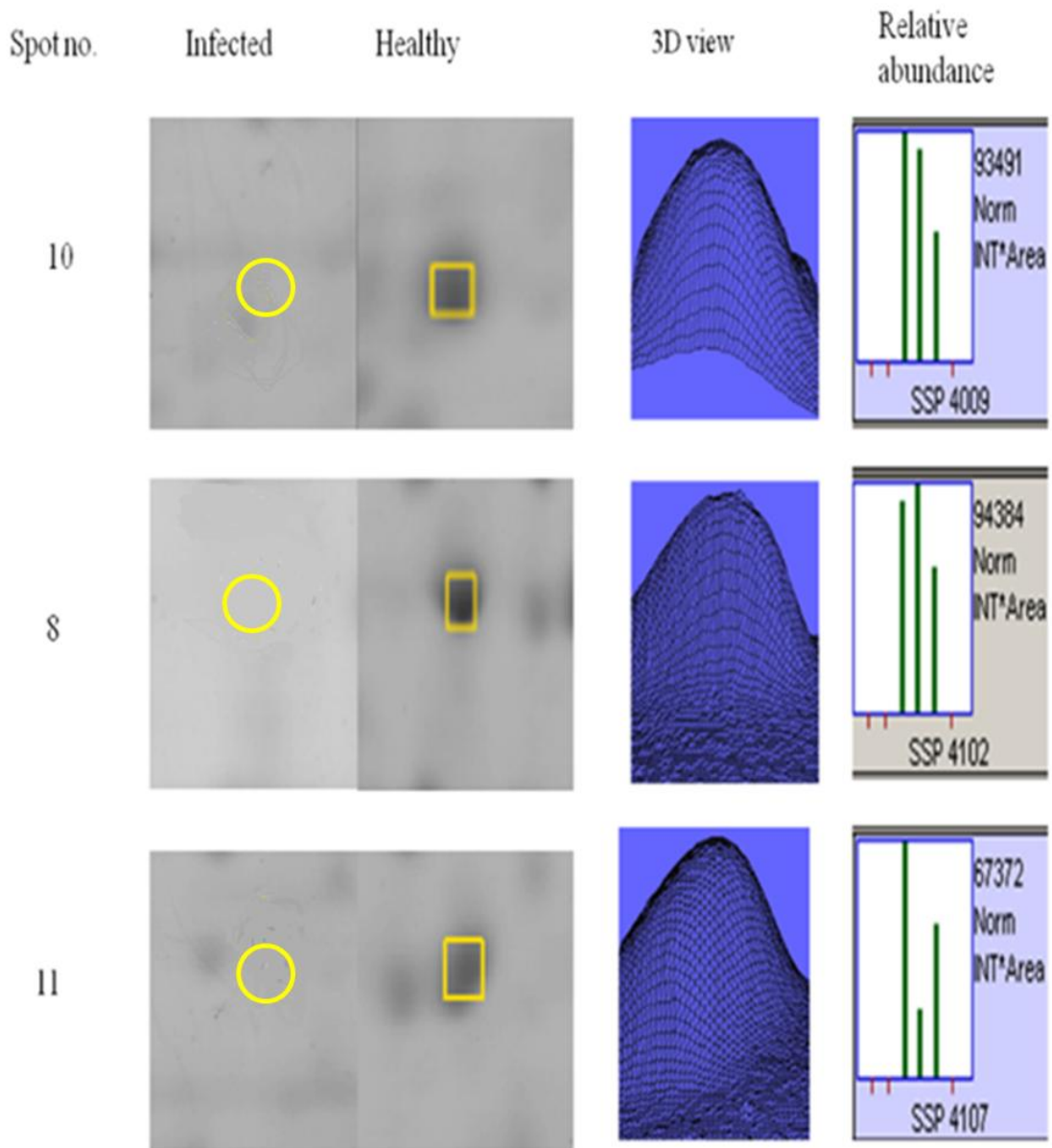


Figure 3.23. Representation of three protein spots only present in 2D gels of leaves of healthy plants (square box) compared to leaves from *F. solani* infected plants (circles) detected using PDQuest software, with spot numbers relating to spots in Fig. 3.22. The 3D view of spots and the relative abundance of the protein in each of 3 biological replicates from leaves of healthy plants is as shown by PDQuest software.

3.2.2.6. Mass spectrometry and data analysis

On average 247 spots were detected from proteins extracted from leaves of healthy pea plants compared to 195 spots detected from leaves of *F. solani* infected plants using the PDQuest software. The 15 protein spots which altered abundance by at least four fold were picked from the gels, digested manually and analysed by mass spectrometry.

Three proteins were not identified using either MALDI- or Q-TOF as the number of peptides that matched were less than the 5 required to give a reliable identification by MALDI-TOF and the 2 required for Q-TOF.

Unknown protein 1 had an observed molecular weight of 47920 Da and isoelectric point of 5.95, unknown protein 2 had an observed molecular weight of 32800 Da and isoelectric point of 4.45, whilst unknown protein 3 had an observed molecular weight of 33100 Da and isoelectric point of 4.10.

The other 12 proteins were identified using Q-TOF mass spectrometry (Table 3.7). Protein 1 was matched to isocitrate dehydrogenase from *Medicago sativa* and had similar predicted and observed iso-electric points, but the observed molecular weight was 5472 Da greater than the predicted molecular weight. Protein 2 was matched to glycerate dehydrogenase from *Cucumis sativus*, but also with the observed molecular weight 4452 Da greater than the predicted molecular weight, with a 0.85 difference between predicted and observed iso-electric points. Proteins 3, 4, 5, 6, 7, 9 and 10 were matched as carbonic anhydrase proteins from pea, with some differences in their predicted and observed molecular weights and iso-electric points (the observed molecular weights and iso-electric points were less than predicted values as shown in Table 3.7). Protein 8 was matched as oxygen-evolving enhancer protein 2 from pea, with the observed molecular weight 6772 Da less than

predicted and a 1.94 difference between predicted and observed isoelectric points. Protein number 11 was identified as matching phosphoglycerate kinase, chloroplastic from *Triticum aestivum*. The observed molecular weight was about 54% of the predicted molecular weight and iso-electric points differed with 2.32. The protein from spot 12 also matched a pea protein, the stromal 70 KDa heat shock-related protein, with almost identical observed and molecular weight values but a 3.68 difference between observed and predicted iso-electric points.

Table 3.7. Proteins that differed in abundance in pea leaves in response to infection of roots by *F. solani*, identified using ESI Q-TOF MS/MS. Spots numbered 1-11 are from healthy and protein 12 from infected plants, as labelled in gels in Fig. 3.22; proteins 1-11 were decreased in abundance by at least four fold, while protein 12 was increased by at least four fold following infection.

Spot no.	Matching protein ¹	Protein accession no.	Organism	Observed/predicted MW(KDa)	Observed/predicted PI	Matched peptides	Sequence coverage %	Score ²
1	IDHP	Q40345	<i>Medicago sativa</i>	54167/48695	6.00/6.15	30	38	1040
2	Glycerate dehydrogenase	P13443	<i>Cucumis sativus</i>	46360/41908	5.10/5.95	28	30	737
3	Carbonic anhydrase	P17067	<i>P. sativum</i>	344730/35640	6.30/7.01	19	39	556
4	Carbonic anhydrase	P17067	<i>P. sativum</i>	29737/35640	5.90/7.01	46	51	754
5	Carbonic anhydrase	P17067	<i>P. sativum</i>	25632/35640	5.30/7.01	51	42	691
6	Carbonic anhydrase	P17067	<i>P. sativum</i>	25632/35640	5.50/7.01	55	37	625

Table 3.7. Continued

7	Carbonic anhydrase	P17067	<i>P. sativum</i>	30684/35640	6.40/7.01	46	51	856
8	OEE2	P16059	<i>P. sativum</i>	21429/28201	6.35/8.29	41	53	856
9	Carbonic anhydrase	P17067	<i>P. sativum</i>	26263/35640	6.70/7.01	17	43	507
10	Carbonic anhydrase	P17067	<i>P. sativum</i>	30684/35640	6.80/7.01	12	48	524
11	Phosphoglyate kinase, chloroplastic	P12782	<i>Triticum aestivum</i>	27210/49980	8.90/6.58	2	8	125
12	HSP70	Q02028	<i>P. sativum</i>	75000/75583	8.90/5.22	38	39	1535

Foot notes

1. IDHP = isocitrate dehydrogenase; OEE2 = oxygen-evolving enhancer protein 2; HSP70 = stromal 70 KDa heat shock-related protein
2. Ion score is $-10 \cdot \log(p)$, where p is the probability that the observed match is a random event. Protein scores are derived from ion scores as a non-probabilistic basis for ranking protein hits.

3.2.3. Root knot nematode (*Meloidogyne hapla*)

3.2.3.1. Pea inoculation

Pea plants were inoculated with 2000 eggs and juveniles (J2 stage) of *M. hapla* strain VW9, maintained using the method of Anita *et al.* (2006) on different tomato cultivars. Reliable infections were obtained on roots of the tomato cultivar Money Maker, which showed a large number of small sized galls (Fig. 3.24) and on pea (Fig. 3.25).



Figure 3.24. Roots of plant of *Lycopersicon esculentum* cv. Money Maker with galls due to infection by *Meloidogyne hapla*. Some are small (*) whilst others merged to give thickened zones (arrows).

The galls of *M. hapla* on the roots of tomato plants ranged from being small and discrete to larger thickened areas (Fig. 3.24). In addition, roots were reduced in size compared to roots of healthy plants (data not shown).

Roots of pea plants infected by *M. hapla* appeared to have no obvious *Rhizobium* nodules (Fig. 3.25). *Rhizobium* nodules on healthy plants were situated on the side of the root and easily detached, whilst nematode galls were an integral part of the root and could not be removed easily. Leaves of infected plants appeared a lighter green colour compared to leaves from healthy plants, and were also reduced in size compared to leaves of healthy plants (see section 3.5).

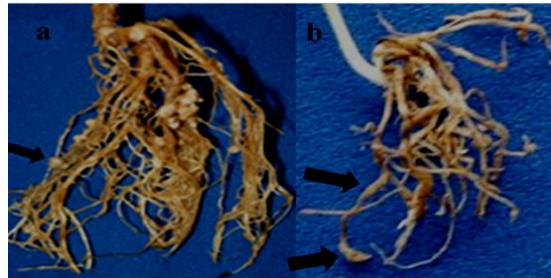


Figure 3.25. Roots of *P. sativum* plants with (a) discrete *Rhizobium* nodules (arrowed) on healthy plants and (b) galls due to infection by *M. hapla* causing thickening of the roots (arrowed).

3.2.3.2. Protein extraction, quantification and purification

The method of Giavalisco *et al.* (2003), referred to here as the Giavalisco method, has been used to extract protein from leaves of healthy and *M. hapla* infected pea plants. It proved to be a suitable method, giving good numbers of protein spots on 2D gels, and clear protein bands on 1D gels.

The effect of *M. hapla* on the amount of protein in leaves of pea plants was studied by extracting proteins from leaves during different stages of infection. The resulting data showed that the amount of protein extracted from pea leaves three weeks after inoculation was significantly less ($p < 0.05$) in leaves from healthy plants ($0.72 \mu\text{g}/\mu\text{l}$) compared to leaves from *M. hapla* infected plants ($0.78 \mu\text{g}/\mu\text{l}$) (Table 3.8). 1D gels also indicated reduction of protein concentration of some protein bands but with increased concentration in others (Fig. 3.26), while no visual difference was noticed when the extracted proteins were separated using 2D gels (data not shown).

Table 3.8. Concentration of protein extracted from leaves of *M. hapla* infected pea plants three weeks after inoculation and leaves of healthy pea plants using the Giavalisco method. Data from 3 experiments, each of 3 biological replicates.

	Replicate	Protein concentration ($\mu\text{g}/\mu\text{l}$)			
		Experiment 1	Experiment 2	Experiment 3	Mean
Healthy	1	0.71	0.68	0.70	0.72
	2	0.74	0.71	0.75	
	3	0.72	0.72	0.72	
	Mean	0.72	0.70	0.72	
<i>M. hapla</i> infected	1	0.77	0.76	0.75	0.78
	2	0.80	0.78	0.78	
	3	0.79	0.77	0.79	
	Mean	0.79	0.77	0.77	

When the proteins were extracted at six weeks post-inoculation, the quantity of proteins in leaves of infected plants appeared to decrease when separated on 2D gels (Fig. 3.27). This was confirmed by protein quantification which showed a reduction in the amount of protein from leaves of *M. hapla* infected pea plants compared to leaves from healthy plants (Table 3.9).

Table 3.9. Concentration of protein extracted from leaves of *M. hapla* infected pea plants six weeks after inoculation and leaves of healthy pea plants using the Giavalisco method. Data from 3 experiments, each with 3 biological replicates.

	Replicate	Protein concentration ($\mu\text{g}/\mu\text{l}$)			
		Experiment 1	Experiment 2	Experiment 3	Mean
Healthy	1	0.52	0.48	0.51	
	2	0.37	0.41	0.40	
	3	0.44	0.48	0.57	
	Mean	0.44	0.46	0.49	
<i>M. hapla</i> infected	1	0.20	0.23	0.30	
	2	0.38	0.29	0.21	
	3	0.30	0.40	0.26	
	Mean	0.29	0.31	0.26	

As mentioned earlier the proteins from pea leaves were extracted as a crude solution. The purification of these proteins was an important step for proteomics because the presence of contaminants will affect the protein quantification and separation when running 1D and 2D gels. Purification of the extracted proteins from pea leaves was required, but this reduced the protein concentration by more than 50% when the 2D Clean-up Kit (GE Healthcare) was used (Table 3.10).

Table 3.10. Concentration of protein extracted from leaves of four weeks old healthy pea plants using the Giavalisco method, in crude extracts and after cleaning with the 2D Clean-up Kit. Data from 1 experiment, with 3 biological replicates.

Extract	Protein concentration ($\mu\text{g}/\mu\text{l}$)			Mean
	Replicate 1	Replicate 2	Replicate 3	
Crude	2.07	5.03	5.01	4.04
Cleaned	1.85	0.96	1.30	1.37

3.2.3.3. 1D-gel electrophoresis (SDS PAGE)

Proteins extracted from leaves of healthy and *M. hapla* infected pea plants three weeks after inoculation were compared by loading the same volume (15 μl) of protein extract from three biological replicates of each onto 1D gels. The results showed that the extracts from leaves from *M. hapla* infected plants gave distinct protein bands in all three biological replicates (see red arrows in Fig. 3.26), with bands from extracts from leaves of healthy plants being less intense. On the other hand, some protein bands (green arrows in Fig. 3.26) appeared to be more intense in extracts from healthy plants. The intensity of bands could have been quantified more accurately by scanning the gels using a Densitometer, but this was not available and a full quantification of the proteins was undertaken using 2D gels. The protein assay data showed that the total amount of protein in leaves of *M. hapla* infected plants is more than the amount of protein in leaves of healthy plants three weeks post-inoculation (Table 3.8). Relative abundance as a result of *M. hapla* infection was investigated further by separation of protein extracts using 2D-gel electrophoresis.

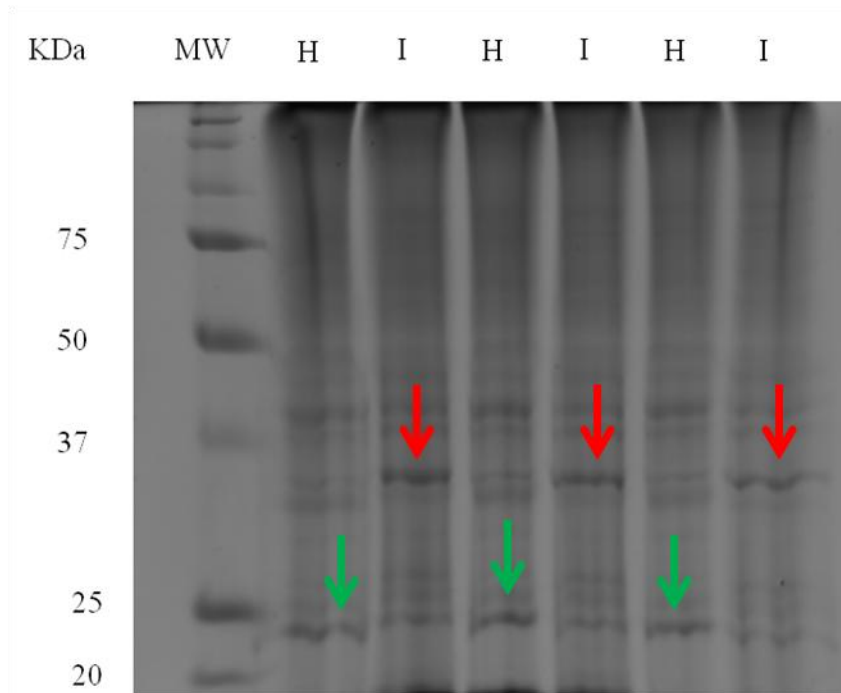


Figure 3.26. Comparison between proteins extracted from leaves of healthy (H) and *M. hapla* infected pea plants (I) using the Giavalisco method, three weeks post-inoculation. Red arrows indicate protein bands with greater intensity in extracts from infected plants and green arrows indicate protein bands with greater in extracts from healthy plants. Three biological replicates; 15 μ l extract loaded in each lane. MW = molecular weight markers, KDa.

3.2.3.4. 2D-gel electrophoresis

Protein samples stored at -80°C were thawed and treated with different procedures including solubilisation, denaturation and reduction, before subjecting to 2D-gel electrophoresis to separate the proteins. Equal amounts of protein extracts from leaves of healthy and *M. hapla* infected pea plants, at three weeks after inoculation from three biological samples, were compared on replicate 2D gels. No visual difference was noticed when the extracted proteins were separated using 2D gels, and this was confirmed by PDQuest analysis (data not shown).

The proteins also were separated using 2D-gel electrophoresis at six weeks after inoculation. The results showed a slight difference in the number of protein spots between extracts from leaves of healthy and *M. hapla* infected plants, and visual observation of the gels indicated the difference in pattern of protein spots between the extracts (Fig. 3.27). Gels were subsequently analysed using PDQuest software, followed by excision and digestion of selected spots, which was then subjected to Q-TOF analysis.

Three gel replicates subjected to PDQuest analysis identified 20 spots that were different in abundance by at least four fold between extracts from healthy and *M. hapla* infected pea plants (Fig. 3.28). Application of a speckle filter, in order to remove noise background and false positive spots, confirmed that these were all protein spots. The PDQuest software normalised the staining intensity of each spot against the sum total of intensities of all detectable spots in the 2D gels, thus correcting minor differences in protein loading or staining intensity among replicate gels. An additional 3D observation of protein spots (Fig. 3.29) showing alteration in relative abundance was performed in order to remove false positives.

1. Healthy

2. Infected

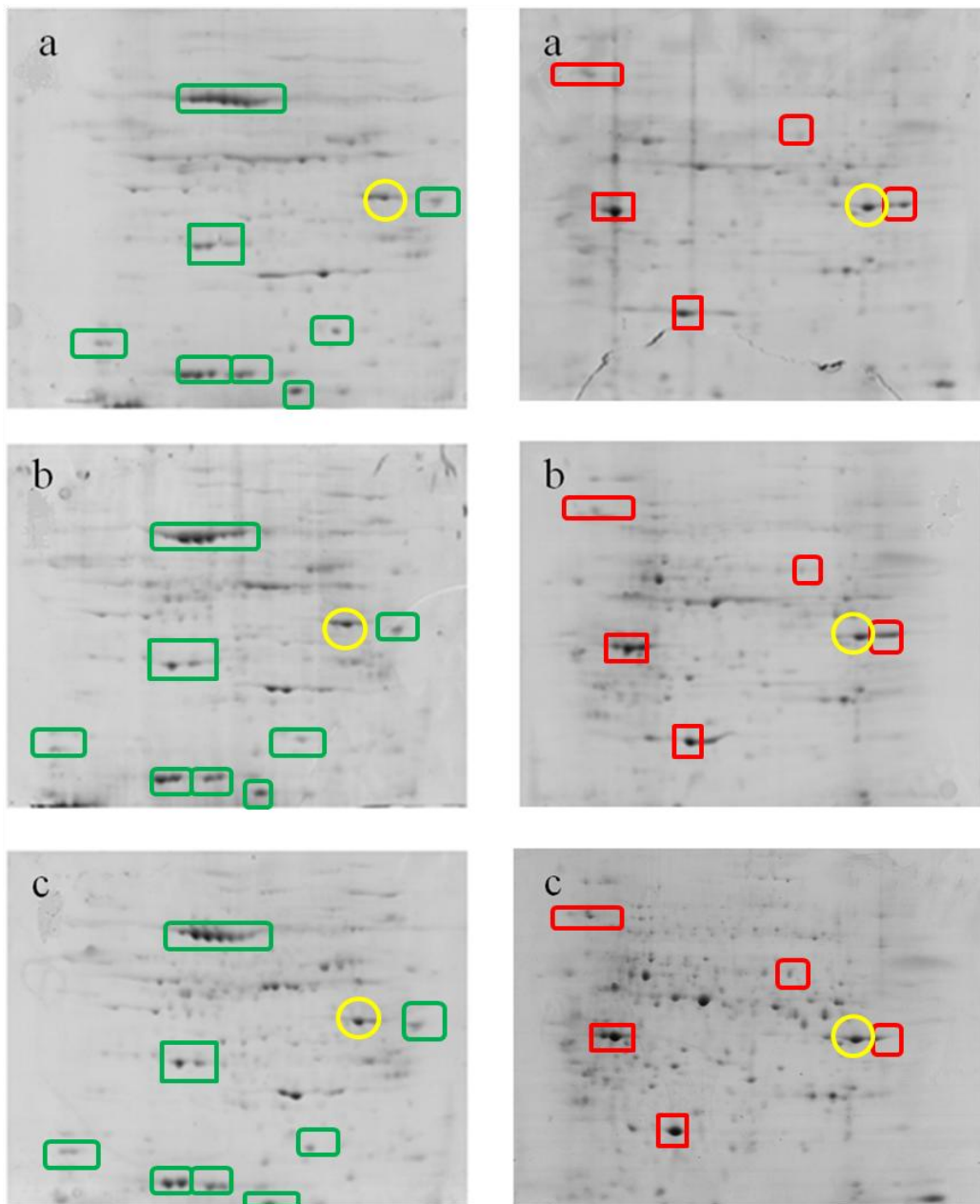


Figure 3.27. Gels of 3 biological replicates of protein extracts from leaves of healthy (1a, b, and c) and *M. hapla* infected (2a, b, and c) pea plants, six weeks after inoculation. Selected proteins identified by PDQuest analysis to be present constantly in extracts from either healthy or infected are indicated in green and red respectively. An example of one protein that is present in all gels is indicated by a yellow circle. Note that some proteins identified as present at the bottom of the gels from healthy samples may have run off the gels of infected samples.

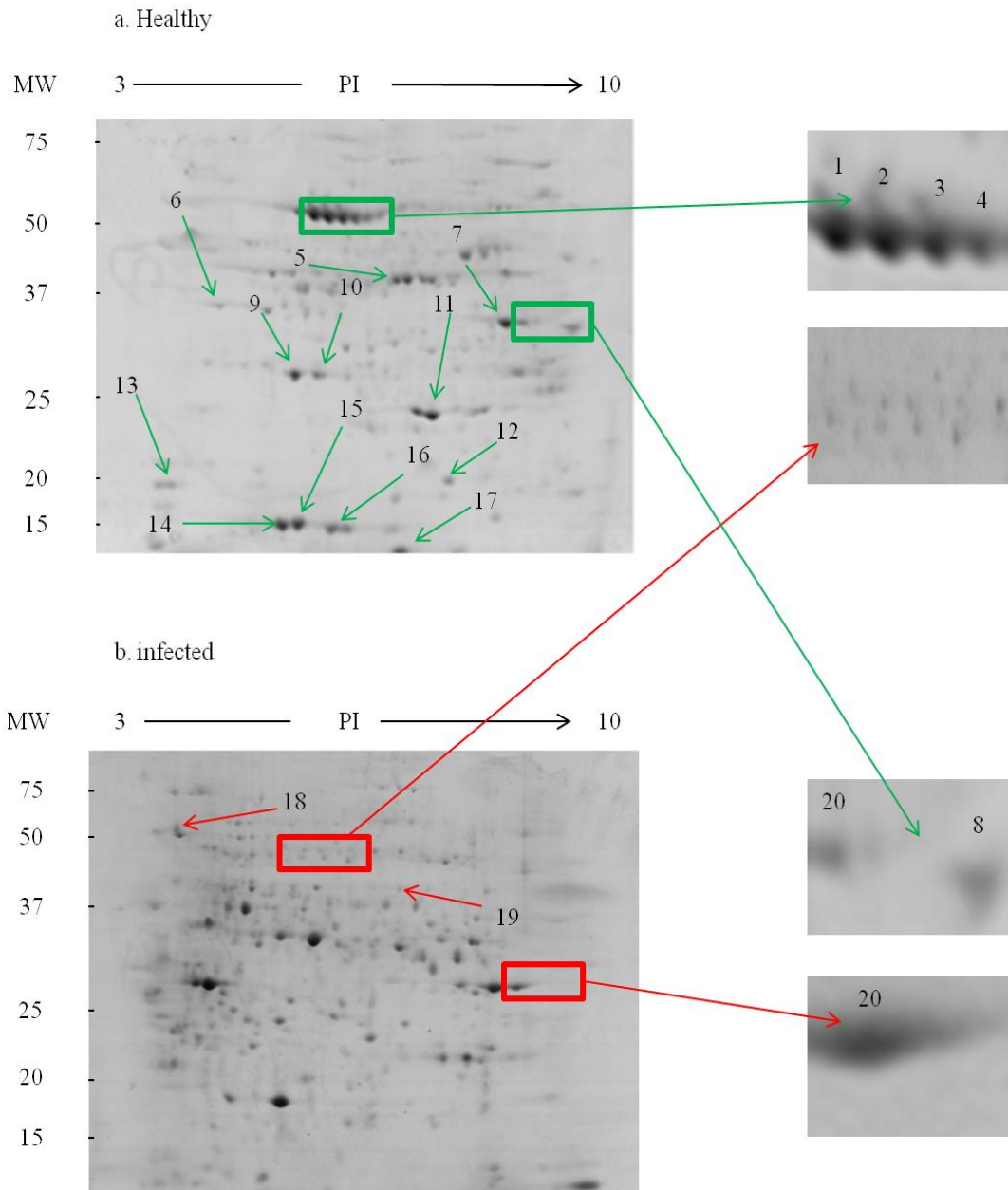


Figure 3.28. Images of 2D gels of proteins extracted from leaves of healthy (a) and *M. hapla* infected (b) pea plants (gels 1c and 2c from Figure 3.27) showing 20 protein spots selected for identification using Q-TOF mass spectrometry. Some samples of identified spots are shown as enlarged images. Spot number is the same as in Fig. 3.29 and Table. 3.11. PI = pH gradient from 3-10; MW = molecular weight (KDa).

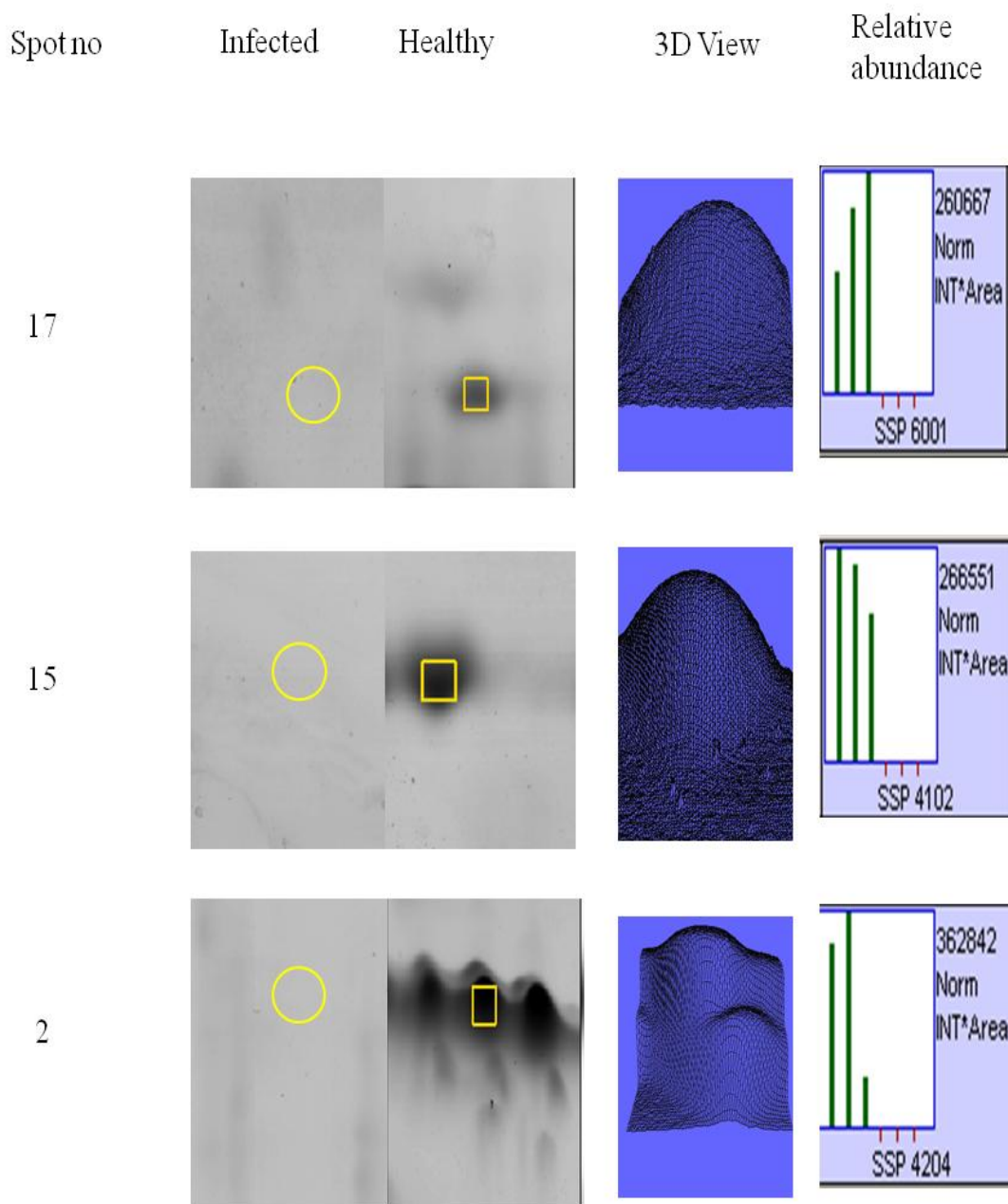


Figure 3.29. Representation of three protein spots only present in 2D gels of proteins from leaves of healthy plants (square box) compared to leaves of *M. hapla* infected plants (circles) detected using PDQuest software. 3D view of spots and the relative abundance of the protein in each of 3 biological replicates from leaves of healthy plants is as shown by PDQuest software.

3.2.3.5. Mass spectrometry and data analysis

On average 120 spots were detected from protein extracted from leaves of healthy pea plants compared to 115 spots detected from proteins extracted from leaves of *M. hapla* infected plants using PDQuest software. Twenty protein spots, with abundance altered by at least four-fold between extracts from leaves of healthy and *M. hapla* infected plants, were separated using 2D gel, digested and analysed by Q-TOF mass spectrometry (Table 3.11).

Seventeen proteins had decreased in abundance following *M. hapla* infection, whilst the other 3 proteins had increased in abundance. In addition, some spots were disregarded as they proved either to be contaminated with keratin or were not real protein spots.

All of these proteins were successfully identified using the Q-TOF mass spectrometry with a high probability score and matched peptides. Proteins 1, 2, 3 and 4 were matched as ribulose biphosphate carboxylase large chain from pea, and shown to have similar observed and predicted molecular weights and iso-electric points (Table 3.11). Protein 5 was matched as fructose biphosphate aldolase 2 from pea, and its observed molecular weight was shown to be 469 Da greater than the predicted molecular weight, and it had a 1.07 difference between predicted and observed iso-electric points. Proteins 6, 9 and 10 were matched as carbonic anhydrase from pea, with protein 6 shown to have very similar observed and predicted molecular weights. For the other two proteins, their predicted molecular weights were greater than the observed molecular weight by 6641 and 8355 Da respectively, and the predicted iso-electric point were greater than observed iso-electric point. Protein 7, 11 and 13 were oxygen evolving enhancer proteins. Protein 7 was identified as oxygen evolving enhancer protein 1 from pea, shown to have

similar observed and predicted molecular weight, with a 2.05 difference between observed and predicted iso-electric points. Protein 11 was identified as oxygen evolving enhancer protein 2 from pea, and its predicted molecular weight was shown to be 3656 Da greater than observed molecular weight, and had a 1.44 difference between predicted and observed iso-electric points. Protein 13 was matched as oxygen evolving enhancer protein 3 from pea, and its predicted molecular weight was shown to be 12670 Da greater than the observed molecular weight, and had a 1.79 difference between predicted and observed iso-electric points. Proteins 8, 12, 16 and 17 were matched as ribulose biphosphate carboxylase small chain from pea, but showed large differences between observed and predicted molecular weights and iso-electric points, except protein 12 which shown to have very similar molecular weight values (Table 3.11).

Proteins 14 and 15 were matched to a 28 KDa ribonucleoprotein from *Spinacia oleracea*, and shown to have a predicted molecular weight 10303 Da greater than the observed molecular weight, but they had similar iso-electric points. Protein 18 was matched as stromal 70 KDa heat shock-related proteins from pea, and shown to have a big difference (25583 Da and 1.62) between predicted and observed molecular weight and iso-electric point. Protein 19 was matched as fructose biphosphate aldolase 1 from pea, and its observed molecular weight was shown to be 3253 Da greater than the predicted molecular weight, with a 0.77 difference between observed and predicted iso-electric points. Finally, protein 20 was matched as trypsin from *Sus scrofa* (Pig), and its observed molecular weight was shown to be 4343 Da greater than the predicted molecular weight, with a 1.20 difference between observed and predicted iso-electric points.

Table 3.11. Proteins that differed in abundance in pea leaves in response to infection of roots by *M. hapla*, identified using ESI Q-TOF MS/MS. Spots numbered 1-17 from healthy and 18-20 from infected plants, as labelled in gels in Fig. 3.28; proteins 1-17 were decreased in abundance by at least four fold, while proteins from 18-20 were increased by at least four fold following infection.

Spot no.	Matching Protein ¹	Accession no.	Organism	Observed/ predicted MW(KDa)	Observed/ predicted PI	Matched peptides	Sequence coverage %	Score ²
1	RuBisCO large subunit	P04717	<i>Pisum sativum</i>	51785/53243	5.35/6.55	31	53	1075
2	RuBisCO large subunit	P04717	<i>P. sativum</i>	50892/53243	5.50/6.55	42	43	998
3	RuBisCO large subunit	P04717	<i>P. sativum</i>	50714/53243	5.80/6.55	47	46	1054
4	RuBisCO large subunit	P04717	<i>P. sativum</i>	50557/53243	6.05/6.55	35	46	1075
5	Fructose – biphosphate Aldolase 2	Q01517	<i>P. sativum</i>	39500/38031	6.55/5.48	32	62	1176

Table 3.11. Continued

6	Carbonic anhydrase	P17067	<i>P. sativum</i>	35685/35640	3.90/7.01	9	35	385
7	OEE1	P14226	<i>P. sativum</i>	33857/35100	8.30/6.25	47	62	1101
8	RuBisCO small subunit	P00868	<i>P. sativum</i>	33612/20402	9.30/9.24	51	55	712
9	Carbonic anhydrase	P17067	<i>P. sativum</i>	28714/35355	5.00/7.01	47	44	704
10	Carbonic anhydrase	P17067	<i>P. sativum</i>	27285 /35640	5.30/7.01	44	49	719
11	OEE2	P16059	<i>P. sativum</i>	24545/28201	6.85/8.29	45	54	960
12	RuBisCO small subunit	P00868	<i>P. sativum</i>	20.000/20402	7.20/9.24	60	51	685
13	OEE3	P19589	<i>P. sativum</i>	19950/32620	3.00/4.79	6	65	159

Table 3.11. Continued

14	28KDa ribonulceoprotein	P28644	<i>Spinacia oleracea</i>	15027/25330	4.65/4.40	3	10	125
15	28KDa ribonucleoprotein	P28644	<i>S. oleracea</i>	15027/25330	4.90/4.40	3	10	125
16	RuBisCO small subunit	P00868	<i>P. sativum</i>	15000/20402	5.50/9.24	46	51	579
17	RuBisCO small subunit	P00868	<i>P. sativum</i>	13000/20402	6.50/9.24	37	52	545
18	HSP70	Q02028	<i>P. sativum</i>	50.000/75583	3.60/5.22	20	27	669
19	Fructose- bisphosphate aldolase 1	Q01516	<i>P. sativum</i>	42000/38747	6.60/5.83	6	25	410
20	Trypsin	P00761	<i>Sus scrofa</i>	29421/25078	8.20/7.00	2	12	104

Foot notes

1. RuBisCO large subunit = ribulose biphosphate carboxylase large chain; RuBisCO small subunit = ribulose biphosphate carboxylase small chain3; OEE1 = oxygen-evolving enhancer protein 1; OEE2 = oxygen-evolving enhancer protein 2; OEE3 = oxygen-evolving enhancer protein 3; HSP70 = stromal 70 KDa heat shock-related protein
2. Ion score is $-10 \cdot \log(p)$, where p is the probability that the observed match is a random event. Protein scores are derived from ion scores as a non-probabilistic basis for ranking protein hits.

3.3. Comparison between proteins extracted from roots of healthy and *M. hapla*, *F. solani* and *P. viciae*, infected plants

Pea root proteins were extracted using the Amey method from different pea plants infected by the different pathogens. The extracted proteins were quantified using the Bradford assay before separating by 1D-gel electrophoresis. The quantification method showed that the amount of protein in roots from healthy plants (0.82 µg/µl) is more than ($n = 3$, $p < 0.05$) the amount of protein extracted from roots of *P. viciae* infected plants (0.75 µg/µl) when compared using one-way ANOVA, whilst it was less than ($n = 3$, $p < 0.05$) the amount of protein extracted from roots from *M. hapla* (2.96 µg/µl) and *F. solani* (1.76 µg/µl) infected plants (Fig. 3.30). The roots infected by *M. hapla* showed the highest protein concentration, followed by the roots infected by *F. solani*.

It is particularly significant that the concentration of protein extracted from roots of healthy pea plants is more than that of protein extracted from roots of plants infected by *P. viciae* ($n = 3$, $p < 0.05$).

In contrast to analysis by one-way ANOVA, Post hoc Fisher analysis with 95% individual confidence intervals and a simultaneous confidence level of 80.40% showed no significance difference between healthy and *P. viciae* infected plants. There was a significant difference, however, between these two treatments and *F. solani* and *M. hapla*. In addition, there was a significant difference between *F. solani* and *M. hapla* infected roots.

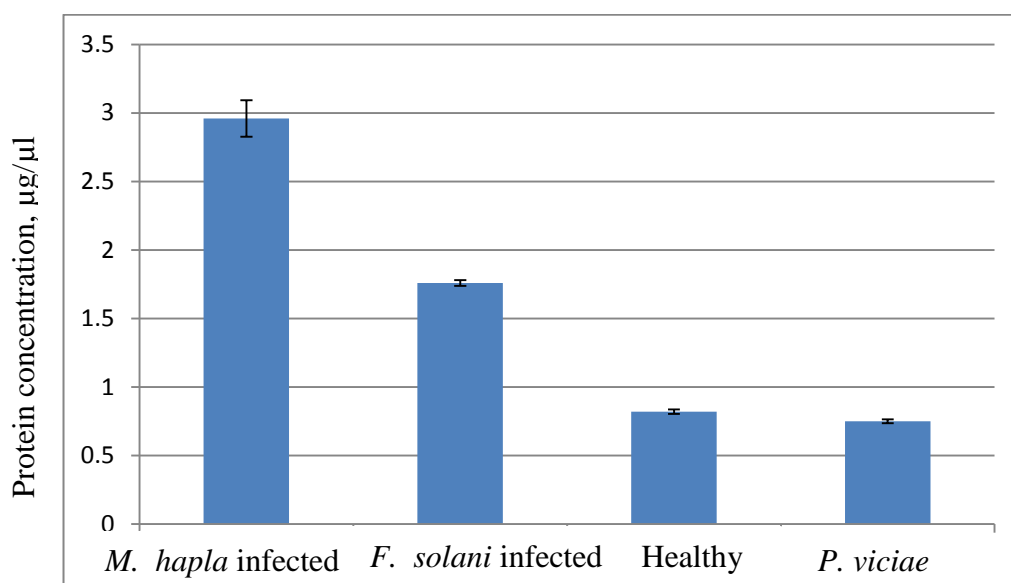


Figure 3.30. Comparison of protein concentrations in extracts from roots of healthy pea plants and plants four weeks after infected by *M. hapla*, *F. solani* and *P. viciae*, using the Amey method (Chuisseu *et al.*, 2007). Data are means of 3 replicate assays of each of three biological replicates (\pm SE).

3.3.1. 1D-gel electrophoresis (SDS PAGE)

All protein extracts were separated by 1D-gel electrophoresis, with the same volume of protein solution (15 μl) being loaded in each lane (Fig. 3.31). The results reflected the data from protein concentration determination, indicating that *M. hapla* and *F. solani* increased the amount of protein in roots compared to healthy plants, whilst *P. viciae* infection decreased the amount of root protein.

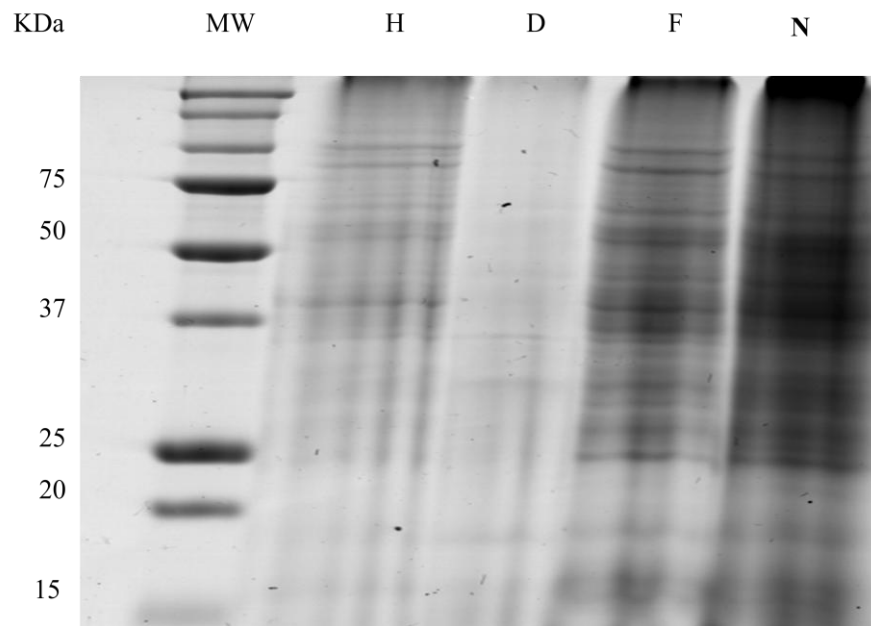


Figure 3.31. 1D gel of proteins extracted from roots of healthy pea plants and plants four weeks after infected by different pathogens (15 μ l loaded in each lane). MW, molecular weight markers; H, roots from healthy plants; D, plants infected by *P. viciae*; F, plants infected by *F. solani*; N, plants infected by *M. hapla*.

3.4. Summary of protein identification: protein biomarkers for specific diseases

Responses of pea plants to infection by the root pathogens *M. hapla* and *F. solani* and the shoot pathogen *P. viciae* were studied using 2D-gel electrophoresis and mass spectrometry.

These studies showed that some proteins increased in abundance in leaves from plants infected by *M. hapla*. The proteins that increased following *M. hapla* infection were HSP70, trypsin and fructose bisphosphate aldolase 1.

Infection of roots by *F. solani* showed that stromal 70 KDa heat shock related protein and two unknown proteins increased in abundance in leaves, and potentially could be used as indicators of infection by *F. solani*.

Whilst several proteins decreased in abundance in roots of plants with *P. viciae* infection, no proteins increased in abundance.

Table 3.12. Summary of proteins identified in pea roots and leaves as a result of infection by *P. viciae*, *F. solani* and *M. hapla*.

Proteins	Healthy		<i>P. viciae</i> , roots	<i>F. solani</i> , leaves	<i>M. hapla</i> , leaves
	roots	leaves			
(1->3)-beta-glucanase	√	X	-	X	X
Alcohol dehydrogenase 1	√	X	-	X	X
Isoflavone reductase	√	X	-	X	X
Malate dehydrogenase	√	X	-	X	X
Mitochondrial ATP synthase subunit alpha,	√	X	-	X	X
Eukaryotic translation inhibition factor	√	X	-	X	X
Superoxide dismutase	√	X	-	X	X
IDHP	X	√	X	-	X
Glycerate dehydrogenase	X	√	X	-	X
Carbonic anhydrase	X	√	X	-	-

Table 3.12 Continued

Proteins	Healthy		<i>P. viciae</i> , roots	<i>F. solani</i> , leaves	<i>M. hapla</i> , leaves
	roots	leaves			
OEE2	X	√	X	-	-
Phosphoglycerate kinase, chloroplastic	X	√	X	-	X
HSP70	X	√	X	+	+
RuBisCO large subunit	X	√	X	X	-
Fructose biphosphate aldolase 2	X	√	X	X	-
OEE1	X	√	X	X	-
RuBisCO small subunit	X	√	X	X	-
OEE3	X	√	X	X	-
28KDa ribonucleoprotein	X	√	X	X	-
Fructose- biphosphate aldolase 1	X	√	X	X	+

Table 3.12 Continued

Proteins	Healthy		<i>P. viciae</i> roots	<i>F. solani</i> leaves	<i>M. hapla</i> leaves
	roots	leaves			
Trypsin	X	√	X	X	+
Unknown 1	X	√	X	-	X
Unknown 2	X	√	X	+	X
Unknown 3	X	√	X	+	X

Abbreviations

√ = present

X = absent

- = decreased following infection

+ = increased following infection

3.5. Effect of *M. hapla* and *F. solani* on pea growth

3.5.1. Leaf area and appearance

The area of leaves from healthy plants was about three times more than from *M. hapla* infected plants, and more than four times more than from *F. solani* infected plants (Fig. 3.32). Likewise, leaves from *M. hapla* infected plants were about 50% larger than from *F. solani* infected plants ($p < 0.05$ for all comparisons between pathogens).

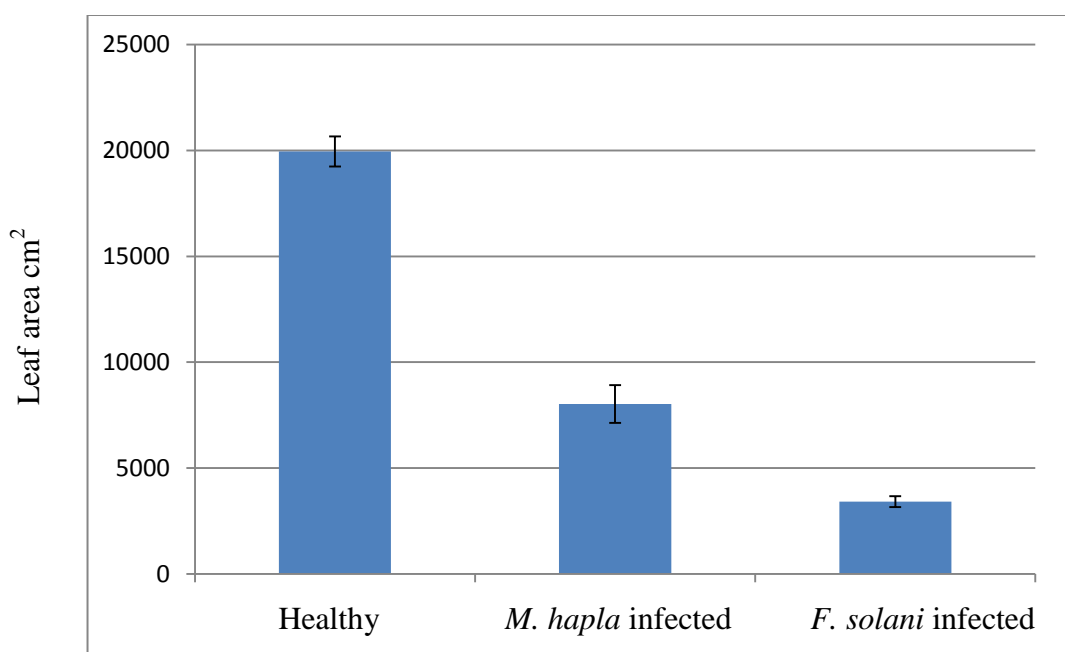
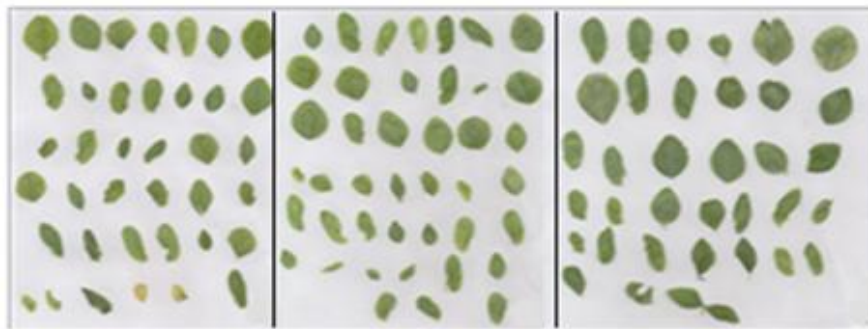


Figure 3.32. Comparison of leaf area of four week-old healthy pea plants and plants infected by *F. solani* and *M. hapla* (n = 3).

1. Leaves from 3 replicate healthy pea plants



2. Leaves from 3 replicate *M. hapla* infected plants



3. Leaves from 3 replicate *F. solani* infected plants

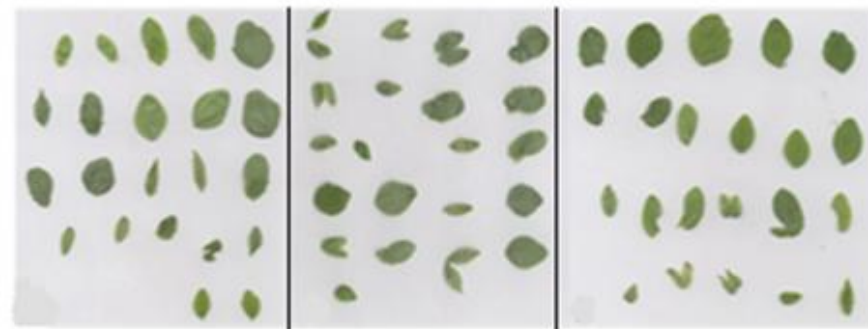


Figure 3.33. Comparison of leaf and stipule size and number from three replicate four week-old pea plants, that are healthy (1) and infected by *M. hapla* (2) and *F. solani* (3).

Healthy pea plants typically had 33 leaves and stipules ($n = 3$), which was not reduced following the infection by *M. hapla*, although the number of leaves and stipules was reduced to 22 due to infection by *F. solani* (Fig. 3.33).

3.5.2. Plant biomass

3.5.2.1. Fresh and dry weight of shoot system

The shoot system of the pea plants four weeks after sowing seeds in these experiments comprised the stem, leaves and stipules. The fresh weight of shoots of pea plants infected by *F. solani* and *M. hapla* were reduced by 66% ($n = 5$, $p < 0.05$) and 56% ($n = 5$, $p < 0.05$) respectively, compared to healthy (control) plants (Fig. 3.34).

The dry weights of the same shoot systems were reduced by 76% ($n = 5$, $p < 0.05$) and 64% ($n = 5$, $p < 0.05$) by *F. solani* and *M. hapla*, respectively (Fig. 3.35).

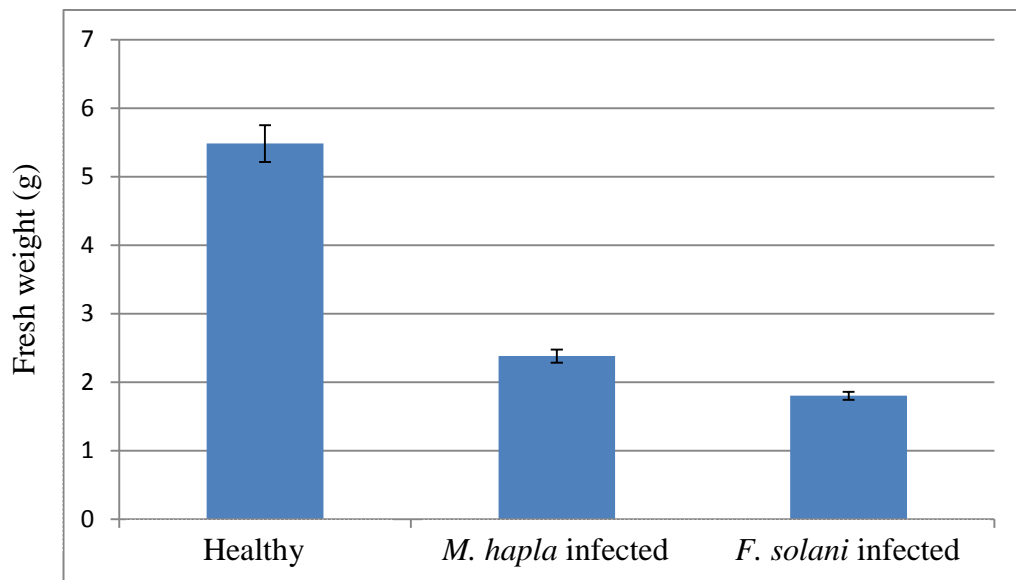


Figure 3.34. Comparison of shoot fresh weights of healthy and *F. solani* and *M. hapla* infected pea plants four weeks after sowing seeds.

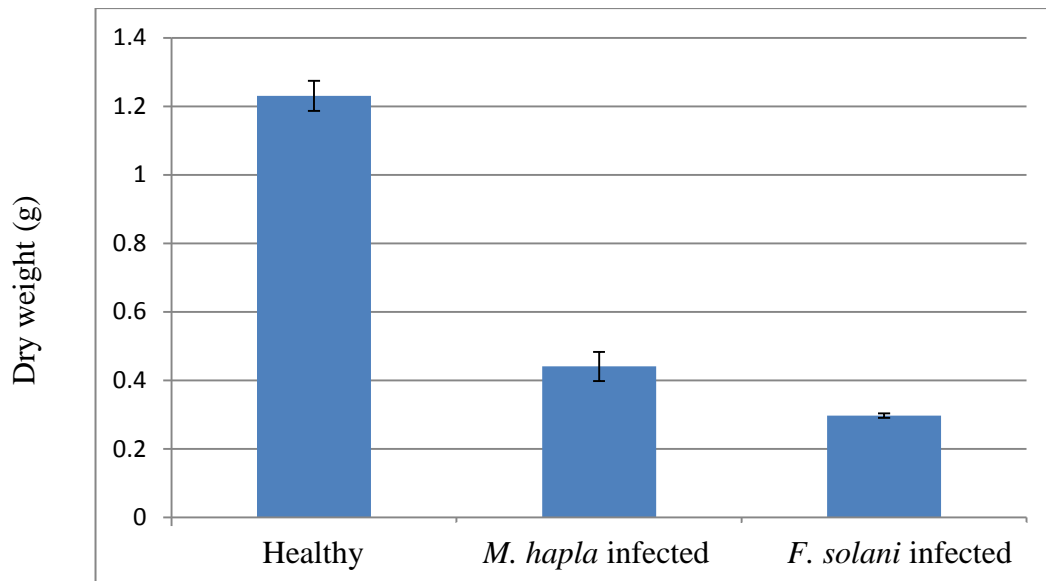


Figure 3.35. Comparison of shoot dry weights of healthy and *F. solani* and *M. hapla* infected pea plants four weeks after sowing seeds.

3.5.2.2. Fresh and dry weight of root system

The fresh weight of roots of pea plants infected by *F. solani* and *M. hapla* were reduced by 80% ($n = 5$, $p < 0.05$) and 20% ($n = 5$, $p < 0.05$) respectively, compared to healthy control plants (Fig. 3.36). The dry weights of the same roots were reduced by 64% ($n = 5$, $p < 0.05$) and 28% ($n = 5$, $p < 0.05$) by *F. solani* and *M. hapla*, respectively (Fig. 3.37).

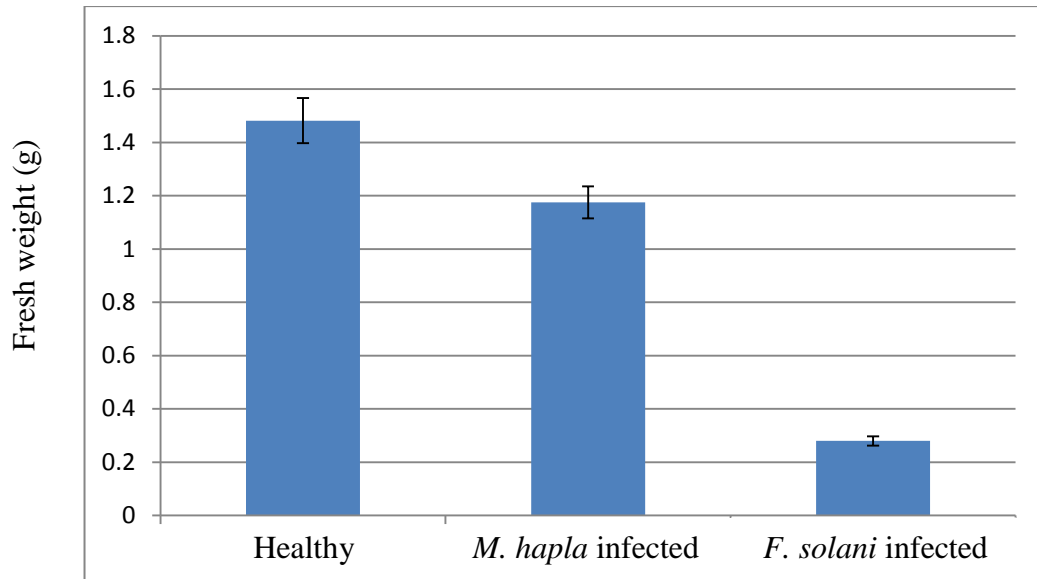


Figure 3.36. Comparison of root fresh weights of healthy and *F. solani* and *M. hapla* infected pea plants, four weeks after sowing seeds.



Figure 3.37. Comparison of root dry weights of healthy and *F. solani* and *M. hapla* infected pea plants, four weeks after sowing seeds.

3.6. Diagnosis of root knot and root rot diseases of peas by processing of leaf images

Pea plants infected by the root pathogens *M. hapla* and *F. solani* superficially showed similar chlorosis and stunting symptoms on the shoot system and therefore are difficult to diagnose and differentiate by the naked eye. In this experiment, two types of camera were used to differentiate between the leaves from plants infected by the pathogens compared to leaves from healthy plants.

3.6.1. Imaging leaves with a digital camera

A clear difference was visible between leaves from healthy plants and leaves from *M. hapla* and *F. solani* infected plants when imaged using a normal digital camera (Fig. 3.38a). Leaves from healthy plants were noticeably larger than those from infected plants, and showed a similar intensity of green colour as those from *F. solani* infected plants. In contrast, leaves from *M. hapla* infected plants appeared a lighter green. After the images had been processed to view the red channel with intensity set at 21% and then superimposed on the original image, the leaves from healthy and *F. solani* infected plants were more clearly distinguished from *M. hapla* infected plants (Fig. 3.38b). When the red channel intensity was increased to 44% (Fig. 3.38c), leaves from healthy plants could be distinguished from those from *F. solani* infected plants.

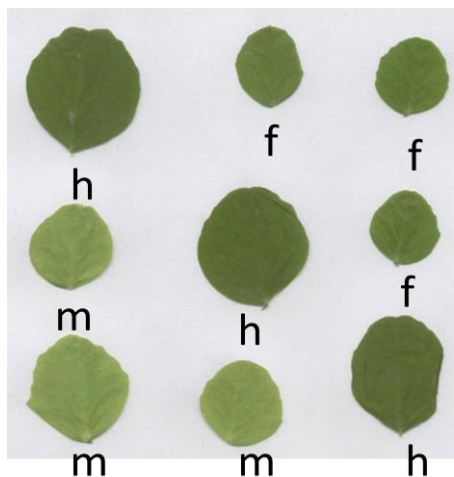


Figure 3.38a. Leaves from healthy (h) and *F. solani* (f) and *M. hapla* (m) infected pea plants imaged with a normal digital camera.

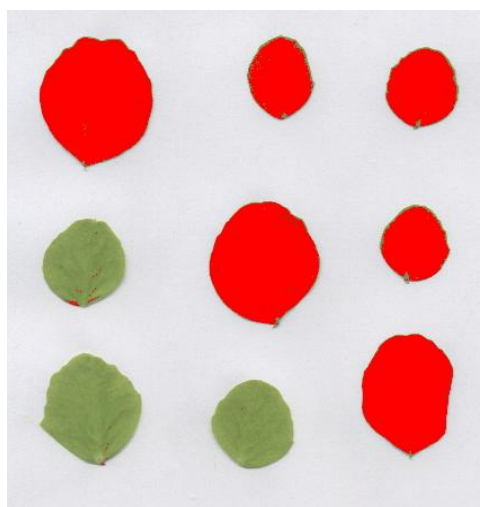


Figure 3.38b. Leaves from healthy and *F. solani* and *M. hapla* infected pea plants imaged as in Figure 3.38a but with the image viewed using the red channel set at 21%, and superimposed on the original image.

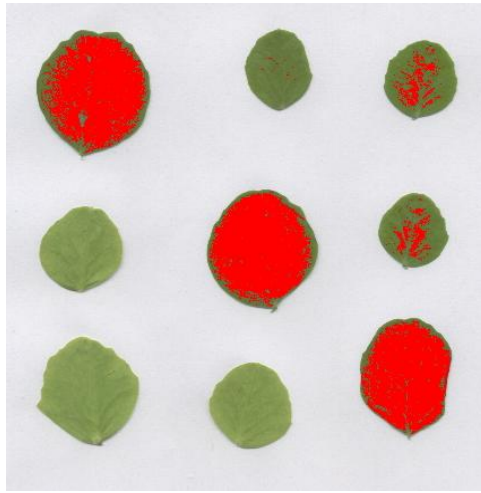


Figure 3.38c. Leaves from healthy and *F. solani* and *M. hapla* infected pea plants imaged as in Figure 3.38b but with the red channel set at 44%.

3.6.2. Imaging leaves with infra-red camera

Images of infected and healthy leaves using an infra-red camera did not distinguish healthy from infected leaves.

4. Discussion

4.1. Inoculation of plants and maintenance of the pathogens

4.1.1. Inoculation with *Peronospora viciae* f. sp. *pisi*

It is difficult to maintain obligate biotrophic pathogens in a non-natural environment, and this makes studying these pathogens a difficult task. Inoculation of pea plants leading to consistent downy mildew infection was the key initial step of this study, where six pea cultivars were inoculated with *P. viciae* (see Materials and Methods) to avoid development of host resistance and to maintain the inocula for other experiments (Ashton, 1994). Subsequently all proteins for proteomics were extracted from one cultivar only (Livioletta) to ensure consistent results.

The optimal conditions for downy mildew were analyzed in previous studies (e.g. Pegg and Mence, 1970). Suitable conditions of light, humidity and temperature as developed by Chuisseu Wandji (2010) were used in the present study, proving to be reliable and ensured a high rate of infection.

4.1.2. Inoculation with *Fusarium solani* f. sp. *pisi*

Inoculation with *F. solani* proved to be easier than with the obligate biotrophic parasite *P. viciae* because *F. solani* is a facultative pathogen and can be grown *in vitro*. Preliminary work compared the two methods of inoculation which had been used by previous workers. The seed inoculation method gave a more reliable infection compared to the soil inoculation method. The reason for this could be that in the seed inoculation method the fungal spores became attached to the seeds before they were sown in soil, so that germ tubes could grow directly into the seeds. As a result, the infection became more severe on the root system, and the roots became brown in colour and more stunted compared to the soil inoculation method. These results agreed with those of Kraft and Kaiser (1993) who found that the

external root colour became dark reddish-brown, especially at soil level and in the cotyledonary attachment area, with greying, yellowing and necrosis of the lower foliage, and stunting when severely infected. In the soil inoculation method, spores would take a longer time to germinate and penetrate the seeds and the chance of infection would be less than in the seed inoculation method. Therefore because the seed inoculation method was easy to apply and gave rapid and reliable infection, it was chosen in this project for all future work.

4.1.3. Inoculation with *Meloidogyne hapla*

It is difficult to obtain sufficient root-knot nematode (*Meloidogyne* spp.) inocula, as either intact egg masses or second-stage larvae, for large experiments. Several disadvantages usually faced when using the egg masses are the time consumed for collecting a suitable quantity of egg masses, and that the egg masses vary in the number of eggs they contain. As a result, roots of inoculated plants usually are not infected uniformly owing to localized concentrations of eggs, and also the egg mass can be contaminated by other pathogenic or saprotrophic organisms. Inoculation with juveniles also causes some problems. With juveniles, the larvae should be physiologically young because infectivity decreases with age and, after inoculation, the optimum environmental conditions must be maintained for larval penetration to occur (Hussey and Barker, 1973). On the other hand, it is not difficult to get infection by *Meloidogyne* spp. in the presence of a susceptible host and suitable inoculum, because root knot nematode larvae enter into roots of tomato plants within 24 hours and giant cell formation starts after 4 days (Bird, 1961). Giant cell formation involves break down of the plant cell walls adjacent to the cell penetrated by the nematode stylet, accompanied by thickening of the remaining cell walls and

an increase in the density of the cytoplasm (Bird, 1961). The number of nuclei within giant cells increases due to mitosis. The protein concentration in giant cells is correlated with the rate of nematode growth (Bird, 1961), and therefore there is likely to be a significant change in the proteome of infected roots.

In this study, *M. hapla* was maintained on different tomato cultivars, with reliable infection of cv. Money Maker which showed high rates of infection and a large number of small sized galls typical of tomato infection (Rahman, 2003). This contrasts with the relatively large galls due to *M. incognita* infection on the same host, which reach to double the size of *M. hapla* galls (Mitkowski and Abawi, 2003). For these reasons, tomato plants make an excellent greenhouse host for the maintenance of most *Meloidogyne* species and have been used frequently as a model system (Mitkowski and Abawi, 2002).

The method of Hussey and Barker (1973) was used to extract eggs from tomato roots, where sodium hypochloride (NaOCl) dissolves the gelatinous matrix surrounding the eggs of root knot nematode (Chitwood, 1938) and surface sterilises the eggs (Loewenberg *et al.*, 1960). The NaOCl method may decrease egg vitality (Chitwood, 1938), but this can be overcome by quick washing of the eggs using water. Certainly in the present study this method did not stop infection occurring.

4.2. Effect of root infection on plant growth

The influence of root pathogens such as *M. hapla* and *F. solani* on pea plants has been estimated by measuring the morphological and physiological characters of infected plants. One of these characters is the leaf area which is considered to be an important parameter for understanding many aspects of functional plant performance (Bunce, 1989). Leaf size is usually related to adaptation of plants and

to the success or failure to establish in a given habitat (Meier and Leuschner, 2008). So the assessment of leaf area can be used to evaluate plant performance at the individual, community or ecosystem level, because the area will affect the production of biomass and the cycling of nutrients in the ecosystem (Meier and Leuschner, 2008). In the present study, Image J software was used to estimate the leaf area of pea leaves to understand the interaction between pea plants and the root pathogens.

M. hapla and *F. solani* have direct effects on plant roots, and therefore these root pathogens affect plant growth and survival (Agrios, 1997). Also root pathogens could have indirect effects on plant growth by affecting the shoot system by preventing or reducing water from being transported to the upper parts of plants. This results in deficiency of water and other nutrients, with leaves becoming a yellow colour and small in size. Thus Abad *et al.* (2003) found that nutrient and water uptake in *Ficus* was substantially reduced because of *M. incognita* infected root systems, resulting in weak and low-yielding plants.

Root knot disease caused by the genus *Meloidogyne* is a complex phenomenon because as well as acting as a metabolic sink, root knot nematodes redirect nutrients within the plant to the root system and elicit profound changes in root morphology (Bergeson, 1966; McClure, 1977; Hussey, 1985). These changes were observed in the present study. The effects of *M. hapla* on pea showed that there was decline in the leaf area as a result of infection, and these results confirm the data of Melakeberhan and Ferris (1989) on *M. incognita* infection of *Vitis vinifera*. Their results showed a decline in the rate of leaf area expansion and leaf, stem, shoot and root weight (excluding nematode weight), but whilst total dry weight of plants

decreased with increasing nematode stress, the root weight including nematodes was not affected.

Infection by *M. hapla* and *F. solani* caused a reduction in the dry and fresh weight of the shoot system of pea plants. This was likely to be as a result of reduced absorbing of water and nutrients via the roots, so less was available for transporting to the shoot system, as well as re-direction of carbon to the roots. The difference in the weight of the shoot system between plants infected by *M. hapla* and *F. solani* compared to healthy plants depended on the severity of infection, with *Fusarium* root rot more severe than *M. hapla* infection and causing a greater reduction in shoot weight. Decrease in photosynthesis occurs soon after inoculation of tomato plants with *M. incognita* and this might explain the reduction of plant biomass accumulation (Fortnum *et al.*, 1991). The photosynthesis rate is thought to be influenced by root knot nematodes because of reduced supply of the root-derived factors such as plant growth hormones (Loveys and Bird, 1973; Wallace, 1974; Melakeberhan *et al.*, 1985; Ahmed and Jehan, 1992). Also, respiration and gross production efficiency decreased significantly with *M. incognita* infection (Melakeberhan and Ferris, 1989). Similar effects are likely in *M. hapla* infected pea plants.

In addition, *M. incognita* has been shown to utilize carbon fixed in the leaf tissue of *Vitis vinifera* cultivars and consume a significant portion of the total energy produced by the plant. The large body size, egg laying capacity and protein content of *Meloidogyne* spp., in addition to modification in the structure and physiology of the plant root, reduce the energy status of the host (Melakeberhan and Ferris, 1988; Melakeberhan and Ferris, 1989), and the alteration in nutrient allocation impacts shoot and root formation (Wallace, 1971). The growth of the shoot system is

therefore suppressed as a result of nematode infection (Wallace, 1971). Indeed the reduced growth of shoot and root systems of pea plants shown here agreed with Santo and O'Bannon (1982), who found that root growth of plants of tomato cvs Columbia, Roza and Salad master was suppressed by infection by *M. hapla*.

A study by Verdejo *et al.* (1988) found that the galls of *M. incognita* on infected roots of *P. sativum* and *Phaseolus vulgaris* developed to contain adult females in about 19 days, compared to 21 to 28 days in this study with *M. hapla*. The difference in life cycle is expected for different species of nematodes. In addition, the rate of development of nematodes inside the roots is affected by temperature, physiological status of the plant host and with changing environmental conditions (Varin *et al.*, 1978).

Roots were smaller and the number of *Rhizobium* nodules decreased on *F. solani* and *M. hapla* infected plants compared to healthy plants. The reason could be that *F. solani* and *M. hapla* infection inhibits formation of nodules on roots by modifying the host response. These results agreed with Verdejo *et al.* (1988), who found that growth of roots and nodules of *P. sativum* and *P. vulgaris* were suppressed as a result of *M. incognita* infection. However, *M. incognita* stimulated the initiation of nodules which remained undeveloped whilst, according to Sharma and Tiagi (1990), the number of nodules on *P. sativum* plants was decreased as a result of infection. They also found that the nitrogen content was reduced in shoots and roots of *P. sativum* infected by *M. incognita*, and therefore an additional consequence of *F. solani* and *M. hapla* infection is likely to be that the amount of nitrogen fixed by roots could be decreased as a result of the decreased number of *Rhizobium* nodules, and this would further decrease shoot growth.

4.3. Effect of age on the amount of protein in pea leaves

The aim of this study was to identify potential protein biomarkers present in leaves that were showing symptoms. As symptoms of *F. solani* and *M. hapla* were first noticed on leaves at 4 and 6 weeks post-inoculation, respectively, these were the ages selected for proteomic analysis.

The results suggested that the amounts of protein extracted after six weeks were less than after four weeks in leaves from healthy plants, using the Giavalisco *et al.* (2003) method. This was confirmed by protein quantification and the average number of protein spots detected using the PDQuest software. Whilst there were 120 protein spots on gels from leaves from plants six weeks old, there were 247 from four week old plants. Similar results were found by Malik and Berrie (1977) who demonstrated that there was decline in certain proteins of pea leaves, with increases in other proteins, as the leaves aged. They also showed that preventing senescence of the whole plant did not alter the pattern of change in leaf proteins.

In addition, Smillie (1962) found that leaves of pea plants at different ages showed differences in their rates of photosynthesis and respiration. The cellular mechanisms controlling these changes are not understood fully, although it is known that they can be influenced by altering the internal or external environment. Smillie (1962) did show, however, that decrease in the rates of photosynthesis or respiration in leaves at certain ages may result from the inability of the leaf cells to fully utilize their potential enzymic capacity, such as transketolase which functions in both photosynthesis and respiration. This might affect the protein synthesis in leaves. These changes also occur in other plant species. For example, Bako (2006) showed the protein concentration of crude extracts from *Zea mays* leaves declined significantly with plant age. In addition, the age of root tissue of *Zostera marina*

had a significant effect on protein metabolism. Therefore the age of leaves and roots is reflected by an altered proteome. This needs to be considered when comparing data of protein from plants infected by different pathogens.

4.4. Protein extraction, purification and quantification

4.4.1. Protein extraction

Protein extraction from plant tissue is a crucial step, because of a relatively low amount of proteins compared to large amounts of different contaminants and interfering compounds such as polysaccharides, phenolic compounds, lipids, organic acids, pigments and other secondary metabolites which pose some problems in protein extraction (Granier, 1988; Shaw and Riederer, 2003; Gorg *et al.*, 2004; Shewry and Fido, 2004; Xu *et al.*, 2008). These interfering compounds influence the resolution of protein separation in 2D gels by causing horizontal and vertical streaking, smearing, and a reduction in the number of distinctly resolved protein spots (Gorg *et al.*, 2004). Therefore developing methods to maximize protein extraction but minimize extraction of interfering compounds was an important first step in this study.

Although the amount of protein in plant tissues is relatively low compared to animal tissue (Granier, 1988), plants still have a wide range of proteins which are different in their characters, so specific conditions are needed to extract and purify these proteins from each species and each tissue. Therefore it is impossible to recommend a single protocol to extract protein from all plant tissue, even in the same species. The presence of cell walls that must be disrupted also makes extracting proteins more difficult (Shewry and Fido, 2004).

As well as causing degradation and protein modification, the interfering compounds have their impact on protein extraction. For instance, salt ions may affect electrophoresis separation, lipids interfere with the extraction of membrane proteins, while polysaccharides and nucleic acids interact with the carrier ampholyte and with proteins, and also increase the solution viscosity (Gorg *et al.*, 2004). The plant proteins are liable to degradation linked to different developmental stages such as differentiation, senescence, and programmed cell death and recent evidence also links proteases with pathogen and stress-induced cell suicide (Beers *et al.*, 2000). These proteases cause the proteolytic degradation of proteins and reduce their molecular weight (Wang *et al.*, 2008).

All these problems were taken in account when selecting methods for extracting proteins from pea tissues. This was facilitated by using one cultivar of pea (Livioletta) for all experiments where proteins were extracted from leaves and roots, which also gave consistent and comparable results.

4.4.1.1. Protein extraction from pea roots

As mentioned earlier, protein extraction is arguably the key step of proteomics, so several protein extraction methods were assessed for pea roots. The common step to extract protein from plant tissue is grinding material using a mortar and pestle in the presence of liquid nitrogen. This practise will break the cell wall and release the cell components, and also reduces proteolysis and any other modes of protein degradation that might occur during the disruption of plant tissue. It produces a very fine powder that gives a greater protein yield (Wang *et al.*, 2003).

Due to the low amount of protein in plant roots compared to leaves as shown in the present study, methods of optimal protein extraction from roots were likely to be

different from those for leaves. Six published methods for pea and other plants have been assessed (see Materials and Methods). These were the Brigham method (Brigham *et al.*, 1995), the Amey method (Chuisseu *et al.*, 2007), the BPP method (Wang *et al.*, 2007), the SDS and SDS acetone methods (Shultz *et al.*, 2005) and the TCA-acetone method (Natarajan *et al.*, 2005).

These methods varied in their efficiency as measured by the quantify of protein extracted. For 1D-gel electrophoresis, the TCA-acetone method (Natarajan *et al.*, 2005) was the best method to extract protein from pea roots, whilst the Brigham method (Brigham *et al.*, 1995) was the least efficient. Significant bands on 1D gels were also obtained by the other methods. The difference between these methods relates to the composition of the extraction buffer and other conditions that were used. In the TCA-acetone method, for example, the pea root powder was dissolved in the TCA-acetone extraction solution compared to lysis buffer that was used in the Amey and BPP methods, and SDS buffer that was used in the other methods. These methods were compared using the published method, without any modification. Thus with the Brigham method (Brigham *et al.*, 1995), only root tips (2.5 cm were used), whilst the other methods used whole roots. This might explain why the Brigham method gave little protein yield, and it would probably give higher yields if all root material was used instead of just the root tips. On the other hand, the Amey method used 100 mg of whole roots and gave a high protein yield compared to the methods that used 100 and 200 mg of plant roots. This study confirmed that the Amey method gave a high protein yield when loaded onto 1D and 2D gels, compared to other methods.

The amount of protein in roots of pea plants infected by *P. viciae* appeared to be less than the amount of protein in roots from non-infected plants, when the protein

extraction was compared by using 1D-gels and the TCA-acetone and the Amey methods. Indeed the protein concentration in the crude extraction solution was approximately 1.13 $\mu\text{g}/\mu\text{l}$ in roots from healthy plants and 0.79 $\mu\text{g}/\mu\text{l}$ in roots from infected plants, when the proteins were extracted using the TCA-acetone method. This compared to 0.59 $\mu\text{g}/\mu\text{l}$ in roots from healthy plants and 0.30 $\mu\text{g}/\mu\text{l}$ in roots from infected plants when extracted using the Amey method (Chuisseu *et al.*, 2007). Whilst both methods provided enough protein to run 2D gels, where the minimum concentration is approximately 0.08 $\mu\text{g}/\mu\text{l}$ (Anon., 2008), the TCA-acetone method gave consistently better yields. The difference between these methods in protein yield might be that in the TCA-acetone method, the protein powder was suspended in TCA-acetone buffer and chilled at -20°C for 1 h, compared to suspension in the lysis buffer used in the Amey method at 4°C .

On the other hand the TCA-acetone method did not show protein spots on separation of the protein extracted using 2D-gel electrophoresis compared to the Amey method which gave a large number of spots. This might be because TCA is a very strong acid and may lead to denaturation and modifications of proteins (Anon., 2012a), which would affect protein separation during the isoelectric focusing that is specific to 2D gels but not part of 1D-gel electrophoresis.

There are several possible explanations for the reduction of protein content in roots from infected plants. Firstly the mechanism of protein synthesis in infected plants could be damaged as a result of infection with the pathogen. Secondly, the pathogen may utilize nitrogen that would be used for protein synthesis in healthy plants. Also, infection of leaves by biotrophic pathogens such as *P. viciae* reduces photosynthesis, and some fixed carbon is utilized by the pathogen instead of being

available for growth of roots (Ayres *et al.*, 1996). This would also reduce the amount of carbon available for amino acid and protein synthesis.

4.4.1.2. Protein extraction from pea leaves

In addition to all the problems mentioned in extracting protein from pea roots, extraction from pea leaves is more complicated because of different contaminant compounds and in particular the constituents of chloroplasts. Chloroplasts contain highly abundant proteins such as ribulose biphosphate carboxylase/oxygenase (RuBisCO) and storage proteins that dominate protein profiles. Due to the diversity of protein abundance, molecular weight, charge, hydrophobicity, post-translational modifications and complexation with other molecules, no single protocol is effective for every protein (Chen and Harmon, 2006).

Two methods of protein extraction from leaves were compared, which were the Amey (Chuisseu *et al.*, 2007) and Giavalisco (Giavalisco *et al.*, 2003) methods. The Giavalisco method gave a large amount of protein compared to the Amey method, and was the optimal method to extract protein from pea leaves. Indeed the Giavalisco method had been used successfully in a previous study of downy mildew infection of pea leaves (Amey *et al.*, 2008). The Giavalisco method was chosen as a suitable protocol to extract protein from pea leaves in all future experiments, because it gave a high rate of protein yield and a good separation of proteins on 2D gels.

4.4.2. Protein purification

Salinity is defined as the amount of salt in water, with conductivity recognized as being the most precise method to measure the salt concentration (Anon., 2010a). All

protein samples were crude extracts, so to avoid any problems during running 2D gels due to salt, protein extracts from pea roots were measured using a conductivity meter which was calibrated using NaCl. The data showed that the amount of salt in protein extract was less than 50 mM equivalent of NaCl, so this amount does not pose any problems in running 2D-gel electrophoresis (Gorg *et al.*, 2004; Anon., 2008), where the maximum recommended is 50 mM.

Non-salt contaminants and interfering compounds in the crude protein extract which may pose problems in running gels (Granier, 1988; Shaw and Riederer, 2003; Gorg *et al.*, 2004; Shewry and Fido, 2004) required a simple and effective method to purify the protein solution before proteomic analysis.

Two methods of protein purification were compared for their effectiveness and simplicity. The Amersham 2D Clean-up Kit, which used three organic reagents for protein precipitation, and the Zeba De-salt Column where resin binds proteins but not other solutes, were compared. After the protein was purified using both methods, equal amounts of both samples were quantified using the Bradford assay (Bradford, 1976). The quantification results showed that the 2D Clean-up Kit caused loss of much protein as a result of the purification steps. This was in agreement with results from Chuisseu Wandji (2010), who found that the 2D Clean-up Kit reduced the amount of protein by approximately 50% when quantified using the Bradford assay. The majority of protein bands which had been detected in 1D gels of the crude extract were visible, but their relative abundance had diminished significantly.

In contrast, samples purified using the Zeba columns did not show any detectable loss of protein when compared to non-purified samples. Indeed some low molecular weight protein bands appeared to be more defined on 1 D gels, however some high

molecular weight protein bands appeared to be less abundant (Chuisseu Wandji, 2010). On the other hand, no clear spots on 2D gels were detectable using protein extracts purified using the Zeba column compared to the 2D Clean-up Kit.

The two methods are different in their procedures; precipitant reagents are included in the 2D Clean-up Kit, whilst no such reagents were used with the Zeba desalt Spin column. The lack of the precipitant reagents may have resulted in permanent protein denaturation during the Zeba purification procedure and this could have affected the protein separation during isoelectric focusing. Based on these results, protein purification using the Zeba column method was ruled out. The 2D Clean-up Kit, however, did not affect the separation of proteins on 2D gels and it was used in all future work to purify the crude protein extract solution. For roots, no clean up method was used because the crude protein extract solution was able to be applied directly for separation on 2D gels, presumably because it contains small amounts of contaminants.

4.4.3. Protein quantification

For 2D-gel electrophoresis sufficient amounts of proteins should be loaded onto each gel, and this is considered to be at least 0.08 $\mu\text{g}/\mu\text{l}$ for 2D gels (Anon., 2008). In this study the Bradford method (Bradford, 1976) was used to quantify proteins before loading onto the gels, and this method proved to be accurate, rapid and simple to apply.

4.5. Comparison of proteins extracted from healthy and infected pea plants

4.5.1. Protein extracted from roots of pea plants infected by *P. viciae*

Proteins extracted from roots of healthy pea plants (control) were compared to protein extracted from roots of plants infected by *P. viciae*. Roots from healthy pea plants contained more proteins compared to roots from *P. viciae* infected plants. The reason is likely to be because *P. viciae* infection of the shoot system affects photosynthesis and the export of photosynthetic products from leaves (Ayres *et al.*, 1996). Some fixed carbon is utilized directly by the pathogen instead of being available for growth of roots (Ayres *et al.*, 1996). This would reduce the amount of carbon available for transport to roots, and therefore affect root growth and the amount of protein synthesis in roots.

There are several other possible explanations for the reduction of protein in roots of infected plants. Firstly, the mechanism of protein synthesis in infected plants would be altered as a result of pathogen infection, a significant change in the proteome after infection has been shown by Amey *et al.* (2008). In their study, several proteins increased in abundance in leaves of *P. viciae* infected pea plants, such as ABR17 stress-response protein, PI176 protein, photosynthetic proteins, a glycine-rich RNA binding protein and glyceraldehyde 3-phosphate dehydrogenases (cytosolic and chloroplastic). A study by Castillejo *et al.* (2010) showed 31 proteins with altered abundance in two cultivars of *P. sativum* with high and incomplete resistance to *Mycosphaerella pinodes*, and these proteins also correspond to those involved in photosynthesis, metabolism, transcription/translocation, defence and stress categories. Although, these identified proteins were different from those identified following *P. viciae* infections, this might be due to the different cultivars and different pathogens, but it is interesting that they have similar functions in plant

metabolism. Secondly, the pathogen may utilize nitrogen that would be used for protein synthesis in healthy plants, which would also reduce the amount of protein in roots.

4.5.2. Protein extracted from roots of pea plants infected by *F. solani*

Roots from plants infected by *F. solani* contained more protein than roots from healthy plants, in contrast to *P. viciae* infected plants. The reason for this might be due to the presence of proteins secreted by *F. solani* in pea roots. Albersheim and Valent (1974) found that microbial pathogens of plants have the ability to secrete proteins which inhibited an endo β 1, 3 glucanase synthesized by the host, an enzyme whose substrate is a constituent of the cell wall of the pathogen. This system was discovered in the anthracnose causing fungal pathogen *Colletotrichum lindemuthianum* and its host, the French bean *Phaseolus vulgaris*. Another possibility might be the presence of host defence proteins as a result of root infection (Veronese *et al.*, 2003). Similar results were obtained by Reddy *et al.* (2005), who found that the amount of protein was increased in roots of *Curcuma longa* infected by *F. solani*. The possible reasons for this could be assessed in future experiments to analyze the proteome of infected roots.

4.5.3. Protein extracted from roots of pea plants infected by *M. hapla*

The amount of proteins in roots from *M. hapla* infected plants is more than the amount of protein of roots of healthy plants, as for *F. solani* infected roots. This is likely to be because *M. hapla* secretes enzymes and other proteins inside the roots and induces formation of giant cells, which causes increased root size (Bellafiore *et al.*, 2008). The root knot nematode is a sedentary parasite (Vovals *et al.*, 2005) and

is present in roots used for protein extraction, so part of the total protein content is nematode protein. Several studies have shown that the amount of protein in galls is more than the amount of protein in healthy root tissue. For example, Trivedi and Tiagi (1986) reported that the galls of *Capsicum annuum* roots infected by *M. incognita* are richer in protein compared to healthy roots, and more protein was noted in tissue near the infection sites. The giant cells showed a gradual increase in the amount of protein in their cytoplasm and nuclei as they developed, becoming maximum as the nematode reaches maturity. In addition, the nematode galls showed increase in lipid content.

4.6. Separation of proteins by electrophoresis

Proteins extracted from pea tissues (leaves and roots) were separated by 1D-gel electrophoresis, which separates protein according to the molecular weight (MW), and 2D-gel electrophoresis that separates proteins according to molecular weight (MW) and isoelectric point (PI).

In 1D-gel electrophoresis the proteins which had been extracted from pea tissues (leaves and roots) gave a number of bands each of which contains several proteins with the same molecular weight (O'Farrell, 1975). On the other hand, two-dimensional polyacrylamide gel electrophoresis (2D PAGE) gives better separation of identified proteins and has become an important technique used in a wide range of various fields in plant biology such as study of analysis of gene expression during development, effects of growth substances, response to stress and the study of polymorphism of proteins (Granier, 1988). It is now the most frequently applied protein separation technique that has been used to analyse proteins, as it has the ability to separate large number of proteins on the same gel (Herbert *et al.*, 1997).

4.6.1. Separation of proteins extracted from roots of pea plants infected by *P. viciae*

Both methods used to extract proteins from roots of *P. viciae* infected pea plants, the TCA-acetone (Natarajan *et al.*, 2005) and the Amey (Chuisseu *et al.*, 2007) methods, gave high protein yields and clear protein bands in 1D gels, although the TCA-acetone method gave better results compared to the Amey method. Separation using 2D-gel electrophoresis, however, showed that only the Amey method gave many protein spots on 2D gels, whilst the TCA-acetone method did not give any spots. The reason might be either the differences in extracting solution used, as already discussed, or alternatively the method was not suitable for extracting proteins from pea roots. The extraction solution of the Amey method was also simpler compared to the TCA-acetone method, and the method took a shorter time to complete. The Amey method also showed a satisfactory protein separation with clear distinct spots on 2D gels, which enabled spot picking for further analysis, and was used for future work.

2D-gel electrophoresis showed that the number of protein spots in gels from roots of *P. viciae* infected plants was less than in roots from healthy plants. Thus there were about 212 from roots of infected plants compared to 229 from root of healthy plants after they were detected using PDQuest software, and this further confirmed that the infection of leaves reduces the amount of protein in roots. This was predicted by the quantification methods, and therefore was not surprising. The reason might be the rate of photosynthesis decreased as a result of infection of pea plants by *P. viciae* (Okorski *et al.*, 2008), and this would have reduced the 12 to 54% of carbon taken in by plants during photosynthesis under the natural conditions that is released to the root system Lynch and Whipps (1990). Some of the carbon

transported to roots stimulates the activity of soil microbes, which are known to promote and stimulate mineral nutrient uptake and transport in the plant (El-Shatnawi and Makhadmeh, 2001). A reduced uptake of minerals would have an impact on photosynthesis, and is also likely to affect the growth and proteome of roots.

4.6.2. Separation of protein extracted from leaves of pea plants infected by *F. solani*

Roots are the main parts of most terrestrial plants for absorbing water and nutrients from soil, and any deficiency in the root system as a result of infection will affect plant growth including the protein content of leaves. The Giavalisco *et al.* (2003) method was used to extract proteins from leaves of healthy and *Fusarium* infected pea plants, then the extracted proteins were separated using 1D-gel electrophoresis.

The quantification data showed that *Fusarium* infection significantly reduced the amount of proteins in pea leaves, and this was reflected in data from 1D-gel electrophoresis. Clear bands were shown when 15 µl of extract from leaves of healthy plants was loaded compared to faint bands when samples from infected plants were loaded.

The difference between proteins extracted from healthy and *F. solani* infected plants was clear, but the gel image (Figure 3.20) was distorted and looks overloaded with regards to the amount of protein. However, the amount of protein used was 15 µl which is suitable for running a 1D gel as the capacity of the gel chambers was 20 µl. One possible reason for the distortion could be that the protein diffused out of

the wells and may have been caused by handling problems when the comb was taken out from the stacking gel, damaging the chambers.

When proteins extracted from leaves of healthy and infected plants were separated using 2D-gel electrophoresis, it showed clear differences in the amount of protein extracted. The number of protein spots on 2D gels of *F. solani* infected plants, was less than in gels of proteins from leaves of healthy plants (control). PDQuest software analysis showed approximately 247 spots in extracts of leaves from healthy plants compared to 195 from leaves of *F. solani* infected plants. The reason might be as described by Pernollet *et al.* (1986) who found that there is relationship between photosynthesis and protein synthesis in Maize. There are strong linear relationships between nitrogen and both RuBP carboxylase and chlorophyll, where the proportion of total leaf nitrogen in the thylakoids remains the same while the proportion in soluble protein increases with increasing nitrogen per unit leaf area (Evans, 1989). In addition, Bethlenfalvary *et al.* (1978) found that the photosynthesis mechanism in *P. sativum* is affected by the nitrogen fixation and this might have a negative effect on protein synthesis in pea leaves.

Thus root infection had a negative impact on leaf protein because it reduced water and nutrient uptake by roots and therefore the growth of leaves. The *Rhizobium* root nodules also had been affected as a result of root infection, which would reduce the amount of nitrogen available for plant growth as discussed in section 4.2.

The gels were analyzed using PDQuest software analysis, before the spots were excised and digested for analysis using mass spectrometry, which is below discussed.

4.6.3. Separation of protein extracted from leaves of pea plants infected by *M. hapla*

Proteins extracted from leaves of *M. hapla* infected pea plants were analyzed by 1D-gel electrophoresis, three weeks post-inoculation. A clear difference in the amount of proteins between leaves from healthy and *M. hapla* infected plants was noted, with 0.78 µg/µl of protein in leaves from infected plants compared to 0.72 µg/µl of protein in leaves of healthy plants. These findings agreed with those of Ahmed and Jehan (1992) in their study on *Lycopersicon esculentum* infected by *M. javanica*. This showed that whilst there were no changes in the amount of protein between leaves of healthy and infected plants after 7-11 days of infection, this was followed by an increase in the amount of protein in infected plants at later stages of infection (14-18 days) and then a decrease compared to leaves from healthy plants from 21 days until the end of the experiment at 45 days. The same results were found by Uritani *et al.* (1971) where the amount of protein in diseased plants decreased in the later stages of infection, but with an increase in free amino acid content. This agreed with the present study where the initial increase of protein concentration in leaves of *M. hapla* infected pea plants was followed by a decrease at six weeks post-inoculation.

On the other hand when the protein extracts were separated using 2D-gel electrophoresis, no clear difference was shown between protein extracts from leaves of healthy and *M. hapla* infected pea plants.

4.7. Identification of proteins

Proteomics is a relatively new field of study for investigating the interaction between diseased plants and their pathogens. 2D-gel electrophoresis was used to

separate proteins before they were stained with Coomassie brilliant blue and analyzed using the PDQuest software. The most abundant protein spots were subjected to further analysis using mass spectrometry either by MALDI-TOF MS or Q-TOF MS/MS, after they were digested manually using trypsin. Robotic digestion was used originally, but it did not give good results in mass spectrometry. The reason might be that the robotic digestion did not digest the protein spots completely, and this is why it gave few peptides which did not represent the protein sufficiently for identification. Subsequently, digestions were undertaken by hand, and this increased the number of proteins given a tentative identification to 100%.

The Mascot software, used to interpret mass spectral data into protein identifications, generates scores that are given a probability value according to how well the observed spectrum matches the theoretical spectrum for a peptide. The threshold score for all peptides identified here had a probability score less than the 5% confidence threshold, indicating that the matches were statistically significant and not simply by chance. However, for a protein to be identified with confidence, the general rule (Anon., 2009) is that at least 5 peptides must be matched from MALDI-TOF, or at least 2 peptides from Q-TOF MS/MS. Therefore proteins that did not match these minimum criteria are not discussed further as regards their identification.

4.7.1. Identification of proteins extracted from pea roots

The 2D gel replicates (Figure 3.14) were quite variable as the different biological replicates were different plants and considerable variability between individual plants would be expected. Optimization of the methods could include refining the fixing, staining and de-staining stages of preparation of gels before analysis. For

example, silver instead of Coomassie staining could be assessed, as silver staining is more sensitive, giving reasonable spots at 50 ng and discernible spots at 5-10 ng compared to Coomassie which gives reasonably dark spots at 1 µg and discernible spots at 100 ng (Anon., 2012c).

Selected protein spots separated by 2D-gel electrophoresis were digested manually with trypsin, and initially the 9 most abundant were subjected to identification using the MALDI-TOF. Any proteins not identified were subjected to further analysis using the Q-TOF, because it is more sensitive compared to MALDI-TOF. Most of the identified proteins matched to pea proteins, but others matched to proteins from *Arabidopsis thaliana* and *Beta vulgaris*.

The score for closeness of matching varied between proteins subjected to MALDI versus Q-TOF mass spectrometry. This was due to the different techniques that were used in matching the peptides mass finger prints (MALDI-TOF) and amino acid sequences (Q-TOF) (Amey *et al.*, 2008), so it is inappropriate to compare the scores of the two methods. Generally the Q-TOF mass spectrometry generates much more information compared to MALDI-TOF, and the maximum score will depend on the mass of the protein, with a greater number of peptides that match giving a higher score. A candidate protein that contains more proteolytic peptides, which can match measured masses, has a higher score. A protein identified often is the protein of highest score (Anon., 2011). In Mascot, the ion score for an MS/MS match is based on the calculated probability, P, that the observed match between the experimental data and the database sequence is a random event. The reported score is $-10 \log(P)$, which gives a higher score when the match is less likely to be due to chance. The protein score in the result report from an MS/MS search is derived from the ion scores. For a search that contains a small number of queries, the

protein score is the sum of the highest ion score for each distinct sequence. The protein score is highly dependent on the information that is in the database used for the search. If a large database is searched, there will be more chance of a match occurring by chance between the experimental results and a protein in the database. This means that the score required for a significant match with a large database (less than 5% probability of a chance match, or 95% confidence) will be higher than when a small database is searched. Previous work (e.g. Amey *et al.*, 2008) has shown that searching for matches in the genome sequence of the pathogens isn't really relevant as most matches will be from pea, and that genome sequence isn't available. Also, the genome sequence of other pathogens was not completely available except the genome sequence of *M. hapla* which is completely identified, but it was not available when proteins were identified in the present study.

All protein spots identified showed a decreased abundance following infection of leaves by *P. viciae*. A possible function of (1->3)-beta-glucanase is in the defence of plants against pathogens, as this protein is constitutively expressed in seedling roots and induced by fungal elicitors. Alcohol dehydrogenase 1 and isoflavone reductase function as oxidoreductase proteins in oxidation-reduction processes, whilst malate dehydrogenase plays a role in the tricarboxylic acid (TCA) cycle. Mitochondrial ATP synthase subunit alpha produces ATP from ADP in the presence of a proton gradient across the membrane, which is generated by electron transport complexes of the respiratory chain. Eukaryotic translation initiation factor 5A-1 plays a role in protein biosynthesis, whilst superoxide dismutase functions in destroying radicals which are normally produced within the cells and which are toxic to biological systems (Expasy, 2010). The possible role of these proteins in the plant's response to infection is discussed in more detail in section 4.8.

Some proteins showed a significant difference between observed molecular weight on 2D gels and the predicted molecular weight, and there are two possibilities to explain this. Firstly, proteins with a low observed molecular weight are likely to be either fragments (Amey *et al.*, 2008), or that they may be a result of post-translational events, such as alternative splicing, endo-proteolytic processing and post-translational modification (Ahmed *et al.*, 2005). Some proteins also showed a difference in their observed and predicted IP. Zhu *et al.* (2005) suggest that this shift is often correlated to protein modification. The modifications that cause such shifts include phosphorylation that can shift the PI by several pH units. For instance, alcohol dehydrogenase 1 is represented by more than one spot with different molecular weight in the present study and these are likely to correspond to isoforms or multiple forms of post-translational modification variants of the same gene product (Castillejo *et al.*, 2010).

4.7.2. Identification of proteins extracted from pea leaves

Pea leaves produce different types of proteins compared to pea roots, because they have different functions. It is also to be expected that proteins in leaves and roots will vary differently in their abundance as a response to infection by different pathogens.

4.7.2.1. Identification of proteins extracted from leaves of *F. solani* infected pea plants

In order to increase knowledge of the pea response to *F. solani*, the leaf proteome was analyzed in healthy and inoculated plants of cv. Livioletta. Under the experimental conditions used, 15 protein spots showed a variance in their

abundance between healthy and inoculated plants. These were subjected to tryptic digestion and analysed by MALDI-TOF first, but this did not result in the reliable identification of any proteins. Thus the spots were subjected to Q-TOF mass spectrometry, because it is more sensitive. This resulted in 12 proteins being identified. These included: isocitrate dehydrogenase which plays a role in the TCA cycle; glycerate dehydrogenase, with a role in the photosynthesis; carbonic anhydrase which is important in photosynthesis by reversibly hydrating carbon dioxide, OEE2, a photosynthetic enzyme; and phosphoglycerate kinase chloroplastic, with a role in the Calvin cycle (Expasy, 2010).

Most of the identified proteins matched pea proteins, and most decreased in abundance following infection. Stromal 70 KDa heat-shock related protein (HSP70), increased in abundance as a result of infection with root pathogens. In different animal and plant living organisms the heat shock proteins are stress proteins present in all organisms at normal temperature and plays vital roles in normal cell function (Lindquist, 1986; Lindquist, and Craig, 1988). The HSP70 family of proteins function in protein folding, assembly, translocation and degradation in many normal cellular processes. They also stabilize proteins and membranes, and can assist in protein refolding under stress conditions and in response to elevated temperature. They can play an important role in protecting plants from stress by re-establishing normal protein conformation, and hence cellular homeostasis and cell function (Lindquist, 1986; Bond and Schlesinger, 1987; Lindquist, and Craig, 1988; Wang *et al.*, 2004).

Two other unidentified proteins increased in abundance and they might be useful as biomarkers for the diagnosis of the *Fusarium* root rot.

Proteins having similar abundance in leaves of healthy and infected plants have not been identified, because one of the aims of this project was to study the changes of the leaf proteome, to use in protein biomarkers.

Some of the identified proteins appeared in more than one spot on 2D gels, with different molecular weights and isoelectric points due to isoforms or multiple forms/post-translational modification variants of the same gene product (Castillejo *et al.*, 2010), as discussed earlier for roots. Proteins in plants and other organisms undergo numerous post-translational modifications, which help to regulate protein function and can alter protein localization. In leaves, for example, several thylakoids proteins are reversibly phosphorylated in response to environmental changes (Van Wijk, 2001).

4.7.2.2. Identification of proteins extracted from leaves of *M. hapla* infected pea plants

Pea plants inoculated with *M. hapla* showed different proteins in their leaves as a response of root infection by this pathogen compared to leaves from healthy plants, when the proteins were extracted and separated by 2D-gel electrophoresis. The reason for this could be that the root infection reduces the amount of protein in leaves by affecting the photosynthetic and protein synthesis mechanisms. The photosynthesis mechanism is affected in *Meloidogyne* hosts following infection (Loveys and Bird, 1973; Wallace, 1974; Melakeberhan *et al.*, 1985). Also according to Sawhney and Webster (1979) synthesis of proteins and plant growth hormones was blocked by nematode secretion. The reduction of photosynthesis associated with *M. hapla* infection was, likely to be because of decrease in some photosynthetic proteins.

Only about 120 spots were detected from protein extracted from healthy pea plants compared to an average of 115 protein spots from leaves of *M. hapla* infected plants. A four-fold threshold was used to select proteins as explained in section 3.2.2.5 above. Of the proteins that had an altered abundance, 20 proteins were identified using Q-TOF mass spectrometry. The majority (17) of the proteins were observed to have decreased consistently by at least four-fold due to infection by *M. hapla*, and these included RuBisCO large subunit, fructose biphosphate aldolase 2, carbonic anhydrase, OEE1, OEE2, OEE3, RuBisCO small subunit and 28KDa ribonucleoprotein. The possible roles of these proteins are in the Calvin cycle, photosynthesis, glycolysis and mRNA processing (Expasy, 2010).

The remaining 3 proteins were observed to increase in abundance consistently during *M. hapla* infection, and were HSP70, fructose biphosphate aldolase 1 and trypsin. The latter matched trypsin from *Sus scrofa*, which might be derived from the trypsin used in digestion of the protein spots because the source of trypsin is porcine pancreas (pers. Comm., Technical Support, Promega).

The proteins that increased in abundance have potential for use as protein biomarkers in the diagnosis of *M. hapla* diseases.

4.8. Role of proteins with altered abundance

Pea plants contain proteins that play different roles in plant physiological and metabolic processes, and the profile of proteins changes with developmental stage and as different mechanisms are required to cope with biotic and a biotic stress. The plant proteome is modified well before symptoms appear on plant tissue (Amey *et al.*, 2008), and therefore some of these proteins can possibly be used to diagnose diseases in the early stages of infection. It is significant, therefore that proteins with

an altered abundance were identified in roots and leaves of pea plants infected by the foliar pathogen *P. viciae* and the root pathogens *F. solani* and *M. hapla*, as summarised in Table 3.12.

All proteins identified from roots of *P. viciae* infected plants were decreased in abundance following infection of leaves, but were not shown to change in abundance in leaves following *F. solani* and *M. hapla* infections of roots. Each protein with an altered abundance is considered in turn below.

(1->3)-beta-glucanase belongs to a family of plant hydrolytic enzymes thought to play a role in defence mechanisms against microbial attack (De Carvalho *et al.*, 1992). This protein may be involved in the defence of plants through its ability to degrade the cell walls of fungal pathogens (Mauch and Staehlin, 1989). In addition, some of the carbohydrates released from the fungal cell walls can elicit other plant defence responses (Ayers *et al.*, 1976; Yoshikawa *et al.*, 1990). Therefore foliar infection by *P. viciae* would appear to reduce the constitutive defence capabilities of roots of infected plants. These enzymes are termed pathogenesis related (PR) proteins, and are characterized by their rapid accumulation in plants reacting hypersensitivity to pathogen interactions. PR proteins were initially identified in tobacco (Gianinazzi *et al.*, 1970; Van Loon and Van Kammen, 1970) where they have been extensively characterized. Other studies have led to the identification of these proteins in many species including both monocotyledonous and dicotyledonous plants (Bol *et al.*, 1990; Carr and Klessig, 1990). (1->3)-beta-glucanase is constitutively present in roots and floral tissues of healthy plants (Felix and Meins, 1987; Lotan *et al.*, 1989; Memelink *et al.*, 1990; Cote *et al.*, 1991), and induction of increased accumulation is the result of transcriptional activation of the corresponding genes (Castresana *et al.*, 1990). Beta-glucanases have been found to

be induced in response to different stress situations such as chemical and hormonal treatments (Mohnen *et al.*, 1985; Vogeli *et al.*, 1988; Van den Bulcke *et al.*, 1989; Memelink *et al.*, 1990) as well as after pathogen infection. A study by Kombrink *et al.* (1988) showed activity of this protein increased rapidly in potato (*Solanum tuberosum*) leaves inoculated with *Phytophthora infestans*, but they did not investigate activity in roots of infected plants. Indeed neither the abundance nor activity of this protein has been studied before in roots of plants with foliar infection, and therefore the reduced abundance shown in the present study is novel.

Alcohol dehydrogenase (ADH) was decreased in abundance in pea roots as a result of foliar infection by *P. viciae*. It catalyzes the reduction of pyruvate to ethanol, resulting in continuous NAD⁺ regeneration. ADH activity is considered essential for the survival of plants during anaerobic conditions (Johnson *et al.*, 1994). A study on *Arabidopsis* by Chung and Ferl (1999) showed that the alcohol dehydrogenase gene is constitutively expressed at low levels in the roots of young plants grown on agar media and the expression level is greatly induced by anoxic and hypoxic stresses. A consequence of *P. viciae* infection of leaves therefore appears to reduce the ability of the roots to respond to these abiotic stresses.

Isoflavone reductase was also reduced in abundance in roots of *P. viciae* infected plants. It is an enzyme of the isoflavonoids biosynthetic pathway, synthesized by a branch of the phenylpropanoid pathway of secondary metabolism. Other branches of this pathway produce flavones, isoflavones, lignin and anthocyanin pigments. Genes encoding many enzymes active in the phenylpropanoid pathway have been identified in many species. However, the gene encoding isoflavone synthase, the first step in the branch of the phenylpropanoid pathway that commits metabolic

intermediates to the synthesis of isoflavones, has proved difficult to identify (Jung *et al.*, 2000).

In plants, isoflavones and isoflavonoids play major roles in the defense response to pathogen attack, and in establishing the symbiotic relationship between the roots of leguminous plants and rhizobial bacteria, which lead to nodulation and nitrogen fixation. In plant defense, they have protective activity against microorganisms and herbivores. For example, in *Medicago sativa* responding to fungal pathogen attack they are synthesized via the isoflavanoid branch of the central phenylpropanoid pathway (Wang *et al.*, 2006). Again, the effect of foliar infection on isoflavone reductase in roots has not been studied before, but reduced abundance is likely to correlate with reduced ability to resist infection by root pathogens.

Malate dehydrogenase (MDH) catalyzes the conversion of oxaloacetate and malate. This reaction is important in cellular metabolism, and is coupled with cofactor oxidation/reduction (Musrati *et al.*, 1998). Malate is a key metabolite in plants, and is involved in numerous processes, including C4 and Crassulacean acid metabolism, photosynthesis, stomatal and pulvinal movement, nutrient uptake, respiration, nitrogen assimilation, fatty acid oxidation and providing energy to bacteroids in root nodules (Vance and Heichel., 1991; Vance, 1997; Miller *et al.*, 1998). Reduced abundance in roots of *P. viciae* infected plants is therefore likely to adversely affect nutrient uptake and the establishment and function of rhizobial N nutrition.

Plants contain several forms of MDH (Miller *et al.*, 1998) and expression of MDH genes is dependent upon function and tissue (Vance *et al.*, 1994; Vance, 1997). For instance, legume root nodules contain specific forms of MDH genes that are expressed 5 to 10 fold higher than in other tissues (Vance *et al.*, 1994; Miller *et al.*, 1998). These isoforms play a crucial role in providing the large amounts of malate

required by bacteria to fix nitrogen and by the plant for assimilation of nitrogen (Vance, 1997). Other isoforms play a critical role in many important metabolic pathways including the tricarboxylic acid cycle, glyoxylate bypass amino acid synthesis, glucoeogenesis and facilitation of exchange of metabolites between cytoplasm and subcellular organelles.

Mitochondrial ATP synthase subunit alpha is located in the inner membrane of the mitochondria, and plays a key role in the energy metabolism of all known organisms (Nowroth, 2003). The complexes of the electron transport chain create a concentration gradient of protons across the membrane, and ATP synthase is then able to utilize this concentration gradient to produce ATP. The reduced abundance of this protein in roots is likely to reduce the synthesis of ATP, and therefore their ability to actively accumulate mineral nutrients in *P. viciae* infected plants. Reduced mineral uptake in turn will reduce growth of whole plant.

Eukaryotic translation initiation factor was also reduced in abundance in pea roots following *P. viciae* infection. It is a highly conserved protein found in all eukaryotic kingdoms. This protein was found to be involved in the development of disease symptoms induced by *Pseudomonas syringae* pv tomato, and regulates programmed cell death caused by infection (Hopkins *et al.*, 2008). Evidence suggests that this protein facilitates specific protein synthesis by promoting nuclear export of specific mRNAs (Bevec and Hauber, 1997). Little is known about how the expression of genes for this protein is regulated, but Wang *et al.* (2001) found that the transcript level increased during natural and stress-induced senescence in tomato. Similar trends were demonstrated in dinoflagellates where transcription of genes for this protein were up-regulated at early G (1) phase of mitosis, which is the period prior to the synthesis of DNA. In this phase, the cell increases in mass in preparation for

cell division. The protein then decreased dramatically on the entry of the S phase of the cell cycle, the period during which DNA is synthesized in the dinoflagellate *Cryptothecodinium cohnii* (Chan *et al.*, 2002). Reduced abundance of this protein might be a consequence of the overall reduction in protein content of roots of *P. viciae* infected plants.

Superoxide dismutase was reduced in pea roots after *P. viciae* infection of leaves, and its role was discussed in section 4.7.1. Reduced abundance is likely to be due to leaf infection, as light enhances synthesis of this protein in plants (Cakmak and Marschner, 1992) and it is probable that the amount of light absorbed by infected leaves was reduced. In contrast, Zacheo *et al.* (1983) found that infection of roots by the nematodes *Heterodera gottingiana* and *Meloidogyne incognita* induced production of this protein in pea and tomato plants.

Overall all, it was not surprising that these proteins were decreased in abundance in roots following infection of leaves, as downy mildew affects photosynthesis and reduces the protein content of pea roots as discussed earlier in section 4.5.1. Also it was not surprising that these proteins did not change in abundance in leaves of pea plants infected by the root pathogens *F. solani* and *M. hapla*. Whilst the main effect on roots of plants with foliar *P. viciae* infections was likely to be reduced availability of photosynthates, root infections are likely to reduce mineral nutrients and water availability to leaves. Further, it is likely that pea plants have different defence mechanisms against different pathogens, especially as the infections were in different plant parts (roots versus leaves). Another factor to be considered is plant age, as the proteins were extracted from pea tissue in different ages and different periods of time after inoculation, as shown in materials and methods.

Most proteins identified from leaves of *F. solani* infected pea plants were decreased in abundance, and these included isocitrate dehydrogenase, glycerate dehydrogenase, carbonic anhydrase, OEE2, phosphoglycerate dehydrogenase, and one unknown protein (unknown protein 1; Table 3.12).

Isocitrate dehydrogenase (IDHP) is an important enzyme in the tricarboxylic acid cycle, which occurs in the mitochondrial matrix. It is responsible for catalyzing the reversible conversion of isocitrate to α -ketoglutarate and CO₂. Eukaryotic cells express three different isocitrate dehydrogenases that catalyze decarboxylation of isocitrate into α -ketoglutarate. Genes that encode these three enzymes are located in the nuclear genome, although their protein products function in the cytoplasm (Jenning *et al.*, 1994) and in plastids (Chen *et al.*, 1989). Decrease of this protein after *F. solani* infection may simply reflect the reduced protein content of leaves.

Glycerate dehydrogenase (GDH) is a member of a family of NAD-dependent dehydrogenases, and plays a role in the glycolate pathway and metabolism of the amino acids glycine, serine and threonine (Expasy, 2010).

It was reduced in pea leaves after *F. solani* infection, which might be due to reduced photosynthesis following root infection, although there is no previous study on the role of this protein in pea plants, some forms of this enzyme such as D-phosphoglycerate dehydrogenase and D-3-phosphoglycerate dehydrogenase were found in pea leaves (Cheung *et al.*, 1968). While the D-phosphoglycerate dehydrogenase was increased in pea plants from the 10th to 17th day after germination, the D-3-phosphoglycerate dehydrogenase remained relatively constant during this period. During etiolation D-phosphoglycerate dehydrogenase decreases in abundance and D-3-phosphoglycerate dehydrogenase activity increased. On the other hand, in apical meristem the level of D-3-phosphoglycerate dehydrogenase

increased more than the D-phosphoglycerate dehydrogenase at all time periods studied. Decreasing levels of both dehydrogenase enzymes were found in epicotyl and cotyledon as the plant aged. Further investigation is needed about whether abundance of this protein is changing either specifically due to *Fusarium* infection or generally in response to any root pathogen. However, it did not change in pea tissue inoculated with *P. viciae* and *M. hapla* pathogens, perhaps reflecting different response mechanisms against different pathogens.

Carbonic anhydrase was decreased in abundance in leaves from plants infected by both *F. solani* and *M. hapla*. It is an abundant soluble protein in the C3 plant chloroplast, but its precise role in carbon assimilation has remained speculative (Majeau and Coleman, 1994). Functionally, the enzyme is capable of rapidly interconverting the major forms of C1 and therefore maintaining the supply of CO₂ for RuBisCo by speeding the dehydration of HCO⁻³ in the stroma (Majeau and Coleman, 1994). The reason for the decrease in abundance in the present study might be because root infection affected photosynthesis due to decreased mineral nutrient uptake, as part of the overall reduced growth of leaves of infected plants.

The role of oxygen evolving enhancer protein 2 (OEE2) was described in section 4.7.2.1. It plays an important role in plant photosynthesis and it is not surprising that this protein decreased in leaves of *F. solani* and *M. hapla* infected plants for the same reasons as for carbonic anhydrase.

Phosphoglycerate kinase, chloroplastic was reduced in abundance in pea leaves following root infection by *F. solani*. This protein occurs in chloroplasts, cytosol and nuclei in higher plants (Anderson *et al.*, 2004). Two phosphoglycerate kinase isozymes were found in the pea leaf (Anderson and Advani, 1970). The chloroplastic enzyme plays an important role in the Calvin cycle (photosynthetic

CO₂ fixation) and in glycolysis. It catalyzes the reaction in the direction of the formation of 1, 3-P2-glycerate, utilizing photosynthetically generated ATP during the day, and can generate ATP in the chloroplast by the reverse reaction at night (Anderson *et al.*, 2004). Again, reduced abundance of this protein probably reflects reduced photosynthetic activity in leaves.

Most proteins identified from leaves of *F. solani* infected plants were not identified in tissue from plants infected by *P. viciae* and *M. hapla*, probably for the reasons discussed above for *P. viciae*.

In contrast, the proteins OEE2 and carbonic anhydrase decreased in abundance in leaves following infection by both root pathogens, whilst HSP70 increased in abundance following infection with both. The genes of the HSP70 family are expressed under a variety of physiological conditions. A number of HSP70 and related proteins present in different cellular compartments and associated with a wide variety of cellular processes have been identified, and studies have revealed biochemical similarities among the related proteins from a single organism as well as among proteins isolated from diverse organisms (Lindquist, 1988). Genetic analysis, which has been carried out only in *E. coli* and lower eucaryotes, showed that HSP70 and related genes are essential for growth either at high temperatures, indicating a critical role in normal cellular physiology for the encoded proteins (Lindquist, 1988). The increase in abundance of this protein was expected as it was identified in previous papers that it increases as a result of response to plant development (Lindquist, 1986). This suggests a common effect of root pathogens on certain leaf proteins, which are therefore not suitable as biomarkers for identifying specific pathogens. In addition, two unknown proteins (2 and 3; Table

3.12) were increased in abundance following infection by the *Fusarium* root rot pathogen.

Most of proteins identified from pea leaves following infection by *M. hapla* decreased in abundance; these included RuBisCo large and small subunits, carbonic anhydrase, fructose biphosphate aldolase 2, OEE1, OEE2, OEE3, and 28KDa ribonucleoproteins. Only two proteins were found to be increased in abundance following infection by *M. hapla*: fructose biphosphate aldolase 1 and trypsin.

RuBisCo large and small subunits play important roles in photosynthesis by catalysing the assimilation of CO₂ by the carboxylation of ribulose 1, 5-biphosphate (Ellis, 1979). RuBisCo has a low turnover number, meaning that relatively large amounts must be present to sustain sufficient rates of photosynthesis (Parry *et al.*, 2007). Furthermore, RuBisCo also catalyses a competing and wasteful reaction with oxygen, initiating the process of photorespiration which leads to a loss of fixed carbon and consumes energy. Additionally, RuBisCo and the photorespiratory enzymes are a major nitrogen store and can account for more than 25% of leaf nitrogen (Parry *et al.*, 2008). The decreased abundance demonstrated in the present study is likely to reflect reduced availability of mineral nutrients, especially N, to leaves of plants with root infections.

Fructose biphosphate aldolase 2, (generally referred to as aldolase 2) plays an important role in plant glycolysis and the Calvin cycle of photosynthetic, carbon fixation, and decreased in abundance following infection by *M. hapla*. This agreed with the study by Abbasi and Komatsu (2004) who found that this protein decreased in abundance in leaves of salt stressed rice plants. More specifically, this agreed with Castillejo *et al.* (2004) who found that fructose biphosphate aldolase was decreased in abundance in pea leaves following infection of roots by the parasitic

plant *Orbanche crenta*. They linked this to a study of transformed potato plants which correlated low levels of aldolase to growth inhibition (Haake *et al.*, 1999), and a similar reason is likely for *M. hapla* infected pea plants.

In contrast, fructose biphosphate aldolase 1 increased in abundance in leaves from *M. hapla* infected plants. The reason for the different effect compared to aldolase 2 is unclear.

OEE1 and OEE3 proteins also play important roles in plant photosynthesis. Thus it is not surprising that these proteins were decreased in abundance following root infection by *M. hapla*, for the reasons suggested for other proteins involved in photosynthesis.

28 KDa ribonucleoprotein was found to be the major RNA-binding protein co-purified during the isolation of the 3[prime] end of RNA-processing activity of several chloroplastic genes in *Spinacia oleracea* (Lisitsky *et al.*, 1995). The expression of chloroplast genes is regulated by a variety of mechanisms, one of which is the modulation of RNA stability during chloroplast development. Chloroplast-precursor RNAs undergo a variety of maturation events including cis- and trans-splicing, cleavage of polycistronic messages, processing of 5' and 3' ends, and editing (Mullet, 1988; Gruissem, 1989; Sugiura, 1991; Rochaix, 1992). Most chloroplast genes contain inverted repeats in their 3' end untranslated region, which are capable of forming stem loop structures and do not serve as efficient transcription terminators. These stem loops are essential for the stability of mRNAs *in vivo* and *in vitro*, and may function as 3' end-processing signals (Stern and Gruissem, 1987; Stern *et al.*, 1989 and 1991). This protein was identified as matching *Spinacia oleracea* as the genome sequence of pea is not complete identified. It decreased in abundance in pea leaves following infection by *M. hapla*,

probably for the reasons explained earlier in relation to the likely reduction in the photosynthetic activity of infected plants.

As discussed in section 4.7.2.2, it was not surprising to find trypsin in the plant samples as it matched to *Sus scrofa* (pig), which is the source of the trypsin used for digestion of proteins excised from the 2D gels. Trypsin is not thought to be a plant protein, and it has not been suggested to play any role in plant root or shoot systems.

4.9. Detection of infection and identification of the cause

4.9.1. Specific protein biomarkers from roots of *P. viciae* infected plants

Pea leaves inoculated with *P. viciae* did not show any increase in abundance of proteins in roots as a result of infection with this pathogen, but did show a reduction in the amount of protein in roots of inoculated plants compared to healthy plants. As already discussed, this might be as a result of a decrease in photosynthesis and export of photosynthates from leaves due to the infection by *P. viciae*, both of which will reduce the amount of fixed carbon transported to roots via the phloem.

Whilst no potential protein biomarker was identified from roots of pea plants following infection by *P. viciae*, this did show that protein levels of pea roots changed following infection by *P. viciae*. This provides evidence that protein levels in roots may be affected by other factors interacting with leaves, such as a result of air pollution or any other contaminants. This would affect the quality of underground crops like tubers, and should be investigated further.

4.9.2. Specific protein biomarkers from leaves of *F. solani* infected plants

Proteins whose abundance was observed to have increased consistently by at least four-fold in leaves of pea plants four weeks after their roots were inoculated with *F. solani* were identified as HSP70 and two unknown proteins were appeared to be specific to *F. solani* infection. Of particular significance is that marker proteins for pea pathogens such *F. solani* may be produced by related pathogens of other crops. Thus it may be possible to develop a generic detection system for early diagnosis that could be used in integrated management strategies to control this important group of plant pathogens.

4.9.3. Specific protein biomarkers from leaves of *M. hapla* infected plants

As for *F. solani* infected plants, proteins whose abundance was observed to have increased consistently by at least four-fold in leaves of pea plants at 6 weeks post-inoculation of roots with *M. hapla* included HSP70, which is therefore not a specific response to either pathogen. Other proteins that increased only after *M. hapla* infections was fructose biphosphate aldolase 1. This may represent a pathogen specific response of the host plant, as there was no evidence that the abundance of this protein changed in response to the other two pathogens.

This does provide proof of principle that pathogen-specific protein biomarkers may be present in leaves of plants with root infections. As mentioned earlier, proteomics provides a method to search for protein biomarkers that could be used in a diagnostic kit for root diseases which are difficult to diagnose by eye. The early diagnosis of disease problems within pea and other crops would have multiple benefits for farmers, consumers, national economies and the environment. As protective and eradicate measures such as fungicides would only be applied when

necessary, the cost to the farmer would be reduced. This would improve profits and make farming more competitive. More specifically, a cheap and sensitive diagnostic tool such as a biosensor would be invaluable for those, such as organic farmers, who are not prepared to use routine, preventative fungicide sprays. Consumers are becoming more aware of pesticides in food, and water pollution from agricultural land poses the biggest threat to rivers in countries such as the UK (DEFRA, 2002). Thus reduced pesticide inputs will aid in developing sustainable agriculture, and may in the future be required by new legislation (Amey and Spencer-Phillips, 2006).

Similarly, this technique can be used to examine the difference in protein levels in response to a variety of non-pathogenic factors, including pesticide treatments and fertilizer application, as well as to compare protein differences between conventional and GM crops. The potential of this proteomics technology to extend the boundaries of crop science generally is therefore considerable, as well as promising new insights into the mechanisms behind plant-pathogen interactions.

4.10. Digital imaging of leaves for diagnosis of root diseases

The traditional methods for diagnosis of plant disease depend on visual inspection of exterior symptoms on the shoot system, and also the destructive rouging of plants to inspect root systems for symptoms. It was noted that leaves from plants infected by different root pathogens had a reduced size and an altered abundance of proteins, so it was considered that the changes might be visible by digital imaging methods. These types of techniques have the potential for early, rapid and remote diagnosis of root disease before visibly noticeable changes of colour and other symptoms appear on leaves. This idea was developed by Charter (1959) who showed that imaging

could differentiate developmental stages of leaves, and therefore might be a useful tool for diagnosis of root diseases in the early stages of infection.

Charter (1959) noted that infrared light is unaffected by the chloroplasts but is highly reflected by the spongy mesophyll tissue. Charter considered this to be important as when plants undergo stresses of many different diseases, one of the first parts of plant to be affected is the spongy mesophyll. These changes occur long before the green colour begins to fade. Since infrared is highly reflected by the spongy mesophyll, and relatively unreflected from the cuticle on the upper surface, changes in the structure or composition of the spongy mesophyll affect the manner in which the infrared light is reflected. So even while the plant continues to look normal to the human eye, infrared imaging was thought to be able to detect the abnormality.

With pea, there were clear differences in appearance between the leaves from healthy and infected plants when using a normal digital camera. Leaves from healthy plants were shown to absorb the red colour more than when other colours (blue and green) were used, after the imaging had been processed by modifying the red colour channel. The reason might be that leaves from healthy plants contain greater amounts of chlorophyll compared to the leaves from infected plants. According to Ahmed and Jehan (1992), infection by the root knot nematode *M. incognita* caused a decrease in the amount of chlorophyll, with greater effect on chlorophyll a than chlorophyll b. In other words there is not only a relationship between absorbance and the amount of chlorophyll, but also the ratio of different chlorophylls, and this would have an effect on the absorbance of visible and infrared light. There is good potential of using this technique in diagnosis of root diseases as it is quick, easy, and rapid technique to diagnose plant disease in the

early stages of infection. This may reduce the amount of pesticides used, and therefore contaminants that could be effect human health and the environment.

Also this technique may allow diagnosis of the changes in leaves due to any deficiency of nutrient and minerals in soil.

When an infra-red camera was used, there was no clear difference between leaves from plants with different root infection. The lack of absorbance at this wavelength is because chlorophyll pigments absorb in the blue (400-500 nm) and red (600-660 nm) parts of the spectrum more strongly than in the green (550 nm) and near infrared (700 nm) regions (Totterdell and Rains, 1973). These data seem to contradict those of Charter (1959), but use of different wavelengths might be worth investigating.

5. Conclusion

Pea plants are exposed to a large number of pathogens which reduce the yield and quality of the pea crop. Plants have a variety of active defence mechanisms that require *de novo* synthesis, resulting in a set of induced defence proteins which might be used as protein biomarkers to diagnose of pea diseases.

The development of reliable methods for pea inoculation with *P. viciae*, *F. solani* and *M. hapla* ensured a supply of plant material for proteomic studies.

Previous studies on pea plants are limited, and the present work has developed methods to extract protein from roots and leaves which was a key step in this project towards enabling proteomic studies. Two methods of protein extraction were selected, the Amey (Chuisseu *et al.*, 2007) and Giavalisco (Giavalisco *et al.*, 2003) methods, to provide high quality protein from pea roots and leaves, respectively, leading to excellent separation profiles on electrophoresis gels. These methods are therefore suitable for further proteomic studies of pea leaves and roots. The responses of pea plants to different pathogens confirmed that the amount of protein in pea roots was reduced following shoot infection, and that root infection reduced the amount of protein in pea leaves. This is expected to cause significant reduction in yield of pea pods and seeds.

No protein biomarkers were identified in roots following shoot infection by *P. viciae*. On the other hand, changes in abundance of specific proteins were identified in leaves as a result of root infection by *F. solani* and *M. hapla*. Selected proteins may have the potential of be used as protein biomarkers to identify causes of root diseases by testing samples of leaves using disposable Diagnostic kits, which are an easy and quick way to diagnose diseases in the field.

Root weight was reduced as a result of infection by *F. solani* and *M. hapla*, because these pathogens suppressed root growth. These effects are likely to reduce water and nutrient transport to the upper parts of plant, and be responsible for the weight, colour and protein changes demonstrated in plant leaves.

Image J software also showed that pea leaves of plants infected by *F. solani* were smaller than those from *M. hapla* infected plants, and imaging of leaves could differentiate between healthy plants and those with roots infected by *F. solani* versus *M. hapla*. This might also be an effective, alternative tool in the diagnosis of root diseases. In contrast, infra-red imaging did not show any difference between leaves from healthy and infected pea plants by both pathogens, and does not seem to be a useful tool in disease diagnosis in the pea crop.

Early detection of plant pathogens would enable controlled use of pesticides only when appropriate and would therefore limit soil and environment contamination as a result of overuse. The possibility of using proteomics to identify protein biomarkers that might be used in a kit to diagnose pea diseases in the early stages of infection was demonstrated.

6. Future work

Proteomic study of pea plants in the present work has provided information for more in-depth future analysis. Methods developed in this project, such as inoculation of pea plants with *Peronospora viciae*, *Fusarium solani* and *Meloidogyne hapla*, in addition to reliable protein extraction protocols for roots and leaves, have opened the way for more studies of the effect of these pathogens on pea plants.

The preliminary proteomic data obtained on these host-pathogen interactions provide the basis for novel investigations of defence mechanisms in pea roots against *F. solani* and *M. hapla*. For example, a proteomic study of enzymes of secondary metabolism and the biosynthetic pathways of antimicrobial chemicals (e.g. phytoalexins), and the abundance of pathogenesis-related (PR) proteins, would potentially add significantly to understanding of the responses of pea roots to these pathogens. In addition, proteins secreted by pathogens within infected roots could be identified, with the timescale of secretion matched to stages of the disease cycle. Description of changes in relative abundance of the secreted proteins would provide new information towards understanding disease processes, potentially leading to novel methods for controlling these pathogens by breeding for resistance.

This study also showed that leaf infection by *P. viciae* reduced the amount of protein in pea roots, but more research is needed to understand the mechanism. Therefore experiments should be designed to determine whether this is due to either a pathogen effect on photosynthesis, and hence reduced carbon transport to roots, or reduced uptake of nitrogen for protein synthesis, or something indirect and more specifically related to the pathogen and host response. It would also be useful to

study the changes in root protein at different stages of leaf infection, in order to better understand the effects observed.

In addition, these results have indicated that proteomics could be used to evaluate the amount and quality of proteins in crops where the harvested part is underground. For example, a proteomic study of the legume *Arachis hypogaea* and the effect of shoot infection, or indeed exposure of shoots to other environmental factors, on the quality of groundnuts might reveal pathogen-specific effects. It would also be of interest to study the protein quality of shoot and fruit crops, following root infection by different pathogens. This is likely to have a negative impact on the amount of protein in shoots and reduce the quality and quantity of the harvested part of the crop.

Some proteins identified in leaves and roots appeared to have different molecular weights to the proteins they matched on the Swissprot database. Further experiments are needed to understand why this is, and whether it is a reflection of the infection of pea tissues. For example, the possibility that this was due to post-translational modification of proteins could be explored by combining different proteomic tools such as liquid chromatography electrospray-ionisation tandem mass spectrometry (LC-ESI-MS/MS) and the MS/MS-interpretation tool to study these changes (Schaefer *et al.*, 2006). Phosphorylation of proteins is a typical post-translational modification which is known to play an essential role in the plant response to pathogens at the level of gene expression and defence signalling (Xing *et al.*, 2002). Analysis of post-translational modification, therefore, will help to understand the biological process of infection and could also provide new tools for the control of pathogens. Further work is also needed to study whether the changes of protein abundance described are similar in different cultivars, to indicate whether

different cultivars produce either the same proteomic response to infection with the same pathogen or that their response is cultivar specific. Little is also known about protein changes in plants as they age, and this should also be included in future studies.

The last part of the present project explored new methods to diagnose root diseases using digital imaging and image processing. Whilst infrared imaging with the wavelength used did not differentiate between leaves of plants with different root pathogens, further work with different wave lengths of infrared light would be valuable. Plant physiological parameters can be assessed using infrared imaging without contact with the plant, and during infection processes such as photosynthesis, transpiration, stomatal conductance, accumulation of salicylic acid and even cell death occur and could be monitored remotely.

The experiments described in this thesis represent the preliminary, proof-of-principle stages of a broader search for new tools that might be used in the diagnosis of plant diseases in the early stages of infection. Once specific protein biomarkers for root pathogens have been found in leaves, this would enable the cloning of genes encoding any proteins shown to be specific for those pathogens. The cloned genes could then be used to transform an expression vector, such as *Escherichia coli*, and used to produce large quantities of the protein for the development of monoclonal antibodies. These antibodies may then be deployed in easy-to-use, rapid and sensitive in-field detection kits for the early diagnosis of these pathogens. One of the advantages of a biomarker-based detection methodology, such as used in the Pocket Diagnostics devices (Amey and Spencer-Phillips, 2006), is that it provides a quick, easy and accurate way for diagnosis of plant diseases in the field. This would be particularly important for diagnosing

pathogens in developing countries, where they cause very substantial crop losses that have a devastating impact on food supply.

7. References

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