Impact of Management Practices on Soil Microflora; Implications for Subsequent effects on Plants and Insects

A thesis submitted by Andrew James Kearsey in accordance with the Requirements of the University of Newcastle Upon Tyne for the degree of Doctor of Philosophy

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Declaration

I declare that no part of this work has been submitted by me for any other degree in this or any other university. All work presented here was conducted by the author, except where otherwise stated in the text

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Thesis Abstract

The perceived benefits of food grown under organic (low-input) management practices include lower levels of potentially harmful pesticides and an increased awareness of environmental protection. It is also believed that such crops are inherently more tolerant to biotic stresses, although there is little evidence to support this. The aims of this thesis are to investigate differential effects of conventional and organic management practices on soil microbial community structure and consequences for plant growth using *Arabidopsis thaliana*, as the model plant species. Soil used in the study was from 'Palace Leas hay plots long term field trial' where Plot 2 is fertilised using farmyard manure (FYM) and Plot 13 is fertilised using inorganic fertilisers. Changes in community structure of the two different fertiliser amendments were measured over time using T-RFLP. The laboratory addition of inorganic fertiliser had a significant effect on the bacterial community ($p=0.047$); however, neither initial field treatment nor time had any effect ($p=0.263$, $p=0.842$, respectively). The fungal community showed no significant effect from field treatment, laboratory amendment or time ($p=0.342$, $p=0.131$, $p=0.219$). *A. thaliana* plants grown in conventionally fertilised soil (Plot 13) grew slower and were smaller than those grown in FYM amended soil (Plot 2). After 28 days, rosette area was 552.1cm^2 and 418.4cm^2 for plants grown with FYM and mineral amendment, respectively ($p=0.01$). Dry weights of the rosette were 92.6mg and 72.8mg for FYM and conventionally grown plants respectively ($p=0.044$). Levels of boron (org=0.73ppm, conv=0.31ppm at 30 days), calcium (org=177.7ppm, conv=124.9ppm, at 30 days), potassium (org=331.0ppm, conv=161.5ppm at 30 days) and magnesium (org=29.5ppm, conv=18.3ppm at 30 days) were all significantly higher in FYM grown plants. The ratios of K:Ca and K:Mg in plants grown on FYM amended soil were higher than for those grown on mineral fertiliser amended soil, indicating that the latter plants were under increased stress. Analysis of the leaf proteome demonstrated that nitrilase 1, 14-3-3 like protein GF14, Heat Shock Cognate 70kDa Protein 1, and glutathione-S-transferases PM24 and ERD13 were up-regulated in conventionally grown plants, whilst lipoxygenase and Annexin D6 were down-regulated. Again, this indicates that *A.thaliana* is under increased stress when grown in conventionally treated soil. Glucosinolates and volatiles, produced as part of the plant's inducible defence system, were investigated by HPLC/MS. There were both qualitative and quantitative differences in glucosinolate profiles. Plants grown on FYM amended soils had higher levels of the alkyl glucoside gluconaprin, whilst those on the conventionally treated soil contained glucoraphanin and glucoerucin. Subsequent aphid infestation resulted in a small (7% and 8% for conventionally and FYM grown plants, respectively) increase in the indole glucosides. Those grown on the conventionally amended soil produced 26 different volatile chemicals compared to 18 from the FYM amended soil (Plot 2); average levels of volatiles released where higher from the former than the latter. The results indicate that there was little effect of amendment practice on direct defence mechanisms in *A. thaliana*, although indirect defence was altered by amendment practice. The interactions between fertiliser amendment, soil microbial community, and plant and aphid herbivores were investigated using Structural Equation Modelling. The only significant relationship was a positive correlation between organic FYM amendment and rate of plant growth ($p=0.004$), indicating that the use of FYM as an additional amendment increased plant growth. The effect of the rate of plant growth on the rate of aphid reproduction was almost significant and negative ($p=0.067$), indicating that the rate of plant growth negatively impacted the rate of aphid reproduction.

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Chapter 1. General Introduction

1. 1. Introduction

1. 1. (1) Changes in agricultural practices since the 'green revolution'

Agriculture in the developed nations changed between the 1940s and the 1960s, in a process known as the "Green Revolution". Large increases in yield of all the major crops were seen during this period, due to advances in several areas. Plant breeding created new varieties of crops, known as the High Yielding varieties (HYVs). These plants had increased potential for uptake of nitrogen, while dwarf traits were also bred in, to prevent lodging, a common problem amongst so called traditional varieties of crops under conditions of high nitrogen.

At the same time, more efficient synthetic forms of fertiliser were being developed and deployed along with more effective pesticides. Agronomic practices shifted from traditional rotations to monoculture and larger fields to allow use of more advanced machinery. In the 40 plus years since 1961, there has been a 12% increase in the global area used for growing crops and a 10% rise in the area under permanent pasture. Yields have increased by 106%, but this is linked to a 638% and 203% increase in the use of nitrogen and phosphorus fertilisers respectively, and a 854% increase in the production and hence use of pesticides (Green et al., 2005).

Figure 1.1 shows the trends in yield increase in the period 1940-2000, as well as the increase in fertiliser application and pesticide production. While the general trend of yield is an upwards increase, the nitrogen-amendment efficiency (annual global cereal production divided by annual global application of nitrogen fertiliser) has rapidly decreased (Figure 1.2) (Tilman et al., 2002). It is believed that these changes have significantly affected the environment and human health.

In the last decade, organic food has become more prominent in supermarkets. Every major supermarket now has a dedicated line of food products, which are certified organic by the Soil Association. These lines cover everything from fresh fruit and vegetables to processed foods such as pizzas. According to the Soil Association, sales of organic food in the UK market were worth £2 billion in 2007.

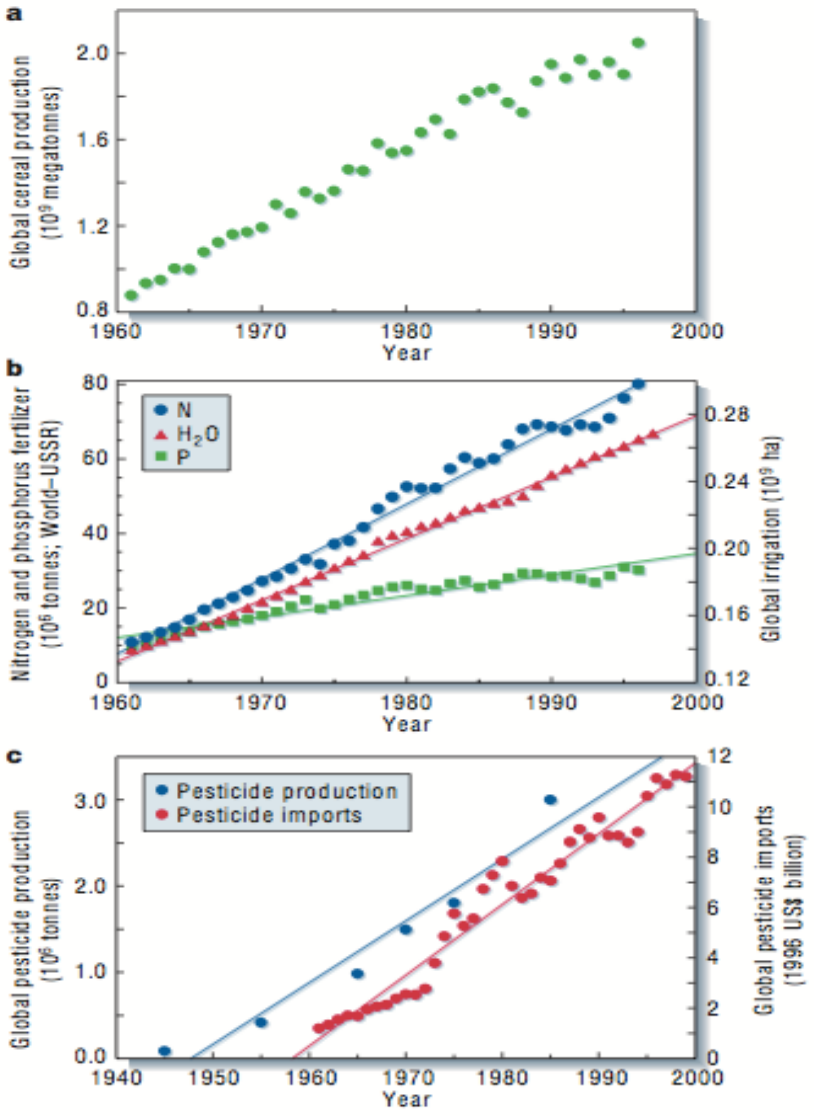

Figure 1.1: Trends in Global Agriculture in the period 1940-2000. a) Total cereal production. b) Total global use of nitrogen and phosphorus fertiliser (excluding former USSR) and area of global irrigated land. c) Global total pesticide production and imports (from Tilman et al (2002))

The market for organic food has increased by 22% between 2005-2006 and the UK is currently the third biggest market for organic food in Europe, after Italy and Germany. However, while there are many claims made about the benefits of organic food, they are often not supported by scientific evidence and analysis.

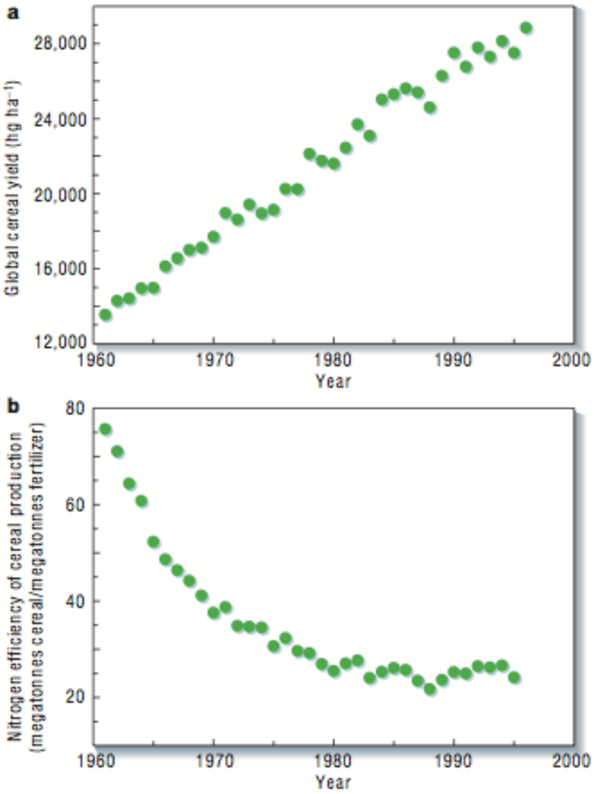

Figure 1.2: Increases in cereal yield are associated with a decrease in the efficiency of nitrogen amendment. a) Trends in average global cereal yields. b) Trends in the nitrogen amendment efficiency of crop production. (from Tilman et al (2002))

1. 1. (2) Definitions of Organic, Biodynamic and Conventional Agricultural Practices

Many field trials and experiments use a comparison of organic practices to conventional practices. Other forms of agriculture, including biodynamic, are also included, for example the DOK (bio-Dynamic, bio-Organic, (K) Conventional) field trial in Switzerland (Mäder et al., 2002).

In the UK, the organic agricultural standards are set by the Soil Association, who have a list of principles and practices that are considered central to the beliefs of organic agriculture. These include using "sustainable" crop rotations, recycling nutrients by using composted manure and vegetable waste, avoiding mineral fertilisers and agrochemical pesticides and using cultivation techniques that protect the soil and its life (Soil Association, 2008). Proponents of organic agriculture aim to treat it as a whole system, that is, from the farm through to the consumer.

Biodynamic agriculture is considered to be the first ecological farming method. It was originally introduced during the 1920s in a series of lectures by Rudolf Steiner (Steiner, 1958). Many of the practices are shared with organic agriculture; indeed the Soil Association acknowledge Steiner's lectures as a founding strand of their movement (Soil Association, 2008). Where biodynamic agriculture differs from organic agriculture is in the use of astrological indicators of when to plant, or as a method of dealing with weed control. There are also various homeopathic remedy analogues used to increase soil fertility. An example of this is the preparation BD501 which is ground quartz, placed in a cow horn and then buried from spring to autumn to allow it to ferment. After this time a small quantity of the dust is mixed in a large quantity of water and sprayed on the crop (Steiner and Smith, 1958). A cow's horn is used, as it is able to channel the "astral life" inwards. However, there is significant controversy over biodynamic agriculture being used in scientific studies. One review of this agricultural practice labelled it "occult and dogmatic and [it] cannot contribute to the development of alternative or sustainable agriculture" (Kirchmann, 1994). In more recent criticism, Trewavas believes that authors who include biodynamic agricultural practices should "either state that they disbelieve the basis of biodynamic farming or justify their occult view" (Trewavas, 2004).

Unlike organic and biodynamic agricultural practices, there is no set definition for conventional practices. Conventional agriculture is best described as the form of management practices that have been introduced using synthetic fertilisers and pesticides. However, many conventional farmers have always used manure as a way of adding nutrients to the soil. More recently, practices such as wide field margins and set aside land have become more common, as these are capable of increasing biodiversity (Leake, 2000).

1. 1. (3) Long term field trials focusing on organic and conventional agricultural practices

There are several significant long term field trials comparing various aspects of organic and conventional agriculture. The longest running is the Broadbalk trial at Rothamsted Research Institute, Harpenden, UK. This trial has been running since 1843. It was originally set up with continuous cropping of wheat, using different levels of nitrogen amendment and farmyard manure. Since then, several changes have been made, mainly during the 1960's to reflect how agricultural practice was changing at the time. On several sections, 5-course rotations were introduced (oats, forage maize, wheat, wheat, wheat) and pesticide use was introduced. On the entire field trial, short-hay cultivars were also introduced and this has continued to be updated to reflect changes within British agriculture (Rothamsted Research, 2006). The Hoosfield long-term experiment at Rothamsted Research is similar to Broadbalk. The Hoosfield trial differs from the Broadbalk trial in that it uses barley as the major crop of interest. This trial was started in 1852 (Rothamsted Research, 2006).

Another long-term UK field experiment is found on the Palace Leas hay plots. This study has been running at Cockle Park Experimental Farm, Newcastle University since 1896. The trial was established to test how amendment practices could regenerate ancient grassland. It was set up as an un-replicated factorial trial, to test farmyard manure and inorganic fertilisers as the treatments (Figure 1.3). Since 1896, the soil microbial and plant communities have been studied and data have been continuously recorded (Shiel, personal communication). The plots used in this study were: Plot 2, which receives farmyard manure as the fertiliser and plot 13 which receives inorganic fertilisers in the form of ammonium sulphate, sodium nitrate, triple superphosphate and potassium oxide (Table 1.1). The average hay yields are 5920t ha^{-1} (±1714) and 4415t ha^{-1} (±1271) for plots 2 and 13, respectively (Coleman et al., 1987; Table 1.2).

Table 1.1. Fertiliser and manure application to each of the Palace Leas Hay Plots, and for plots 3 to 5 the materials applied rotationally by year. Plot 6 receives no amendment (From Shiel, personal communication)

Plot No.	**Rotation year**	**Farm Yard Manure (t ha^{-1})**	**Amendment (Kg ha^{-1})**		
			N	**P_2O_5**	**K_2O**
1		20	17	30	34
2		20			
3	1	20			
	2		17	30	34
4	1	20			
	2				
5	1	40			
	2		17	30	34
	3		17	30	34
	4		17	30	34
6					
7			35		
8				60	
9					67
10			35	60	
11			35		67
12				60	67
13			35	60	67
14			100	66	100

Table 1.2. Hay Yields from each Plot of the Palace Leas Hay Plots (From Coleman et al 1987, Shiel, Personal Communication)

Plot	Hay Yield Kg ha^{-1} (Standard Deviation)	Coefficient of Variation %
1	6496 (2063)	32
2	5920 (1714)	29
3	5142 (1419)	28
4	4824 (1558)	32
5	5120 (1584)	31
6	2554 (1072)	42
7	3049 (1179)	39
8	3463 (1014)	29
9	2317 (1028)	44
10	4113 (1331)	32
11	2928 (1231)	42
12	3873 (1287)	33
13	4415 (1271)	29

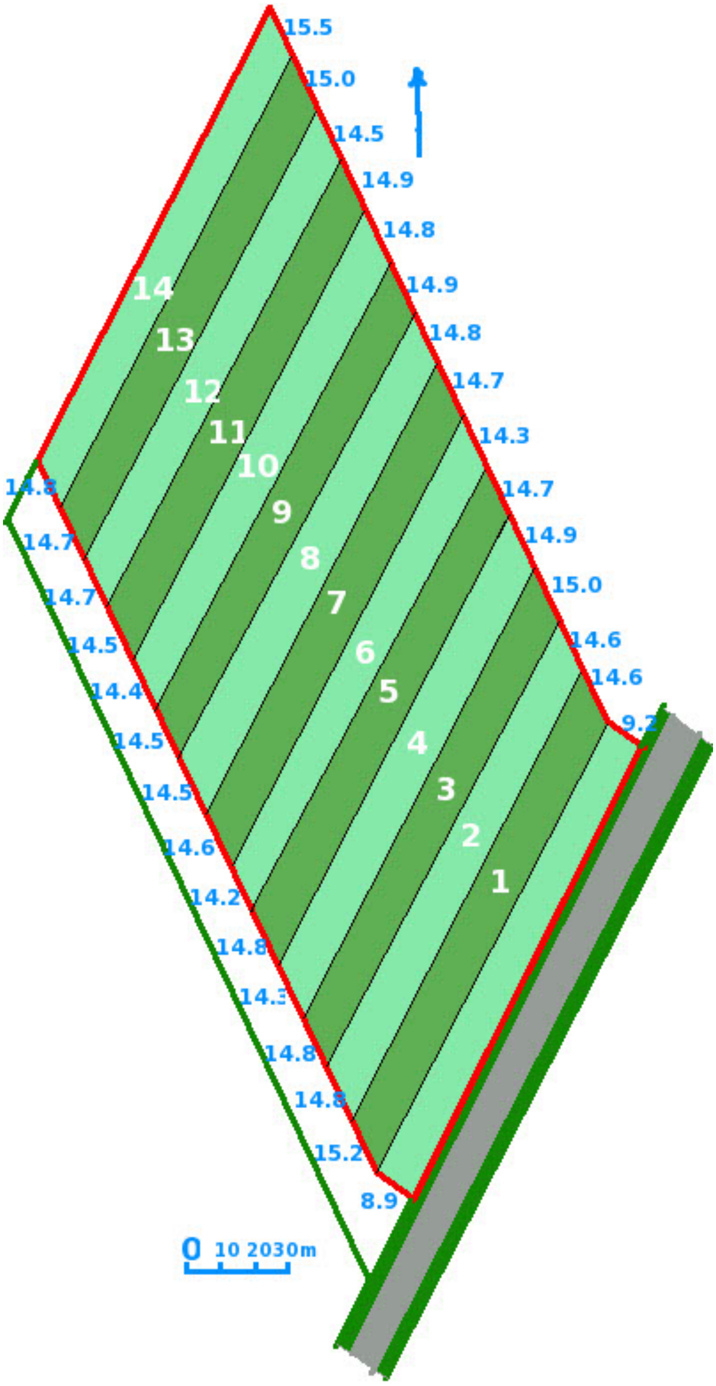

Figure 1.3. The layout of the Palace Leas Hay Plots at Cockle Park Experimental farm (Grid Reference: NZ 201 915). (From Shiel, Personal Communication.) Fertiliser application regimes for the different plots are as stated in Table 1.1.

Of other long-term, classical experiments, there are currently four in North America, four in Europe (excluding Rothamsted) and four in Australia (Rasmussen et al., 1998).

1. 2. Soil fertility and structure as affected by agricultural practices

One of the central tenets of organic agriculture is that it improves soil fertility and structure through the application of farmyard manure (FYM), rather than inorganic fertilisers. Soil fertility has been defined as the ability of a soil to provide the conditions needed for plant growth (Stockdale et al., 2002). The authors provide a detailed analysis of how nutrient profiles and fertility processes differ in organic and conventional agriculture. The conclusions drawn are that nutrient management in organic agriculture is considerably different from conventional agriculture. Nutrients are added via organic sources, such as FYM, or slow release agents, such as rock phosphate, while conventional techniques use highly soluble fertilisers. The organic forms of amendment need time to be broken down into useable components, unlike nutrients that come from soluble fertilisers. It is possible that these soluble nutrients "bypass" the soil processing systems. However, the underlying processes of nutrient processing in soil are the same in both organic and conventional farming (Stockdale et al., 2002).

Nitrate runoff into water supplies is considered a serious problem. It is believed that the use of soluble nitrogen fertilisers would make this situation worse. However, the amount of nitrate lost on organic farms is similar to losses from conventional farms with the organic farms losing about 20% less. The average nitrate loss for organic farms is 47kg/ha, while conventional farms averaged 58kg/ha (Stopes et al., 2002). It is concluded that this is likely to be caused by the longer rotational intervals between cereal crops on organic farms. On matched farms during the ley periods, organic farms lost 45kg/ha nitrate, and conventionally grown grass fields (receiving less than 200kg/ha fertiliser) 44kg/ha indicating that organic farming techniques do not prevent leaching of nitrates (Trewavas, 2004).

Continuous application of minerals does not seem to damage soil fertility in anyway, as evidenced from the Broadbalk study. Some plots have continuously received inorganic fertilizers with no ley periods, other sections have been under the same cropping regimen, except amended with FYM. Yields between the two have stayed comparable over the course of the experiment (Figure 1.4). This indicates that conventional agriculture using soluble mineral fertilisers is a sustainable method of farming (Trewavas, 2004).

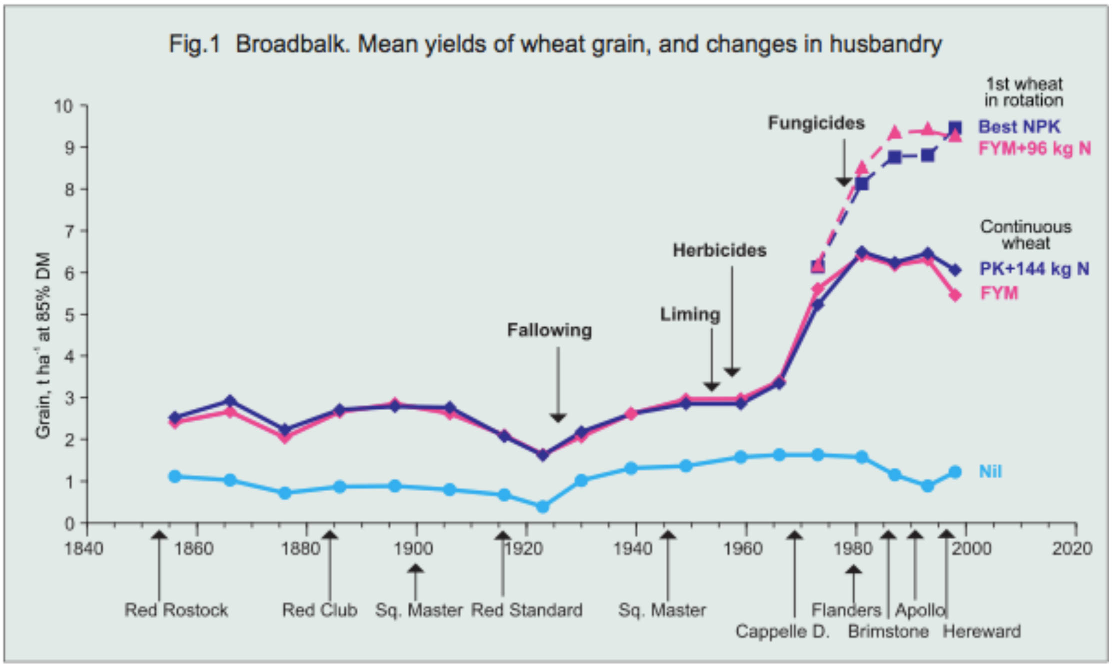

Figure 1.4: Graph showing the changes in wheat grain yield on the Broadbalk field trial. Changes in techniques used are marked on the graph while changes in cultivar are marked on the X-axis. (From Rothamsted Research 2006).

Mineral content in soils needs to be maintained as each crop will remove a large quantity from the soil when harvested. To maintain yields, these losses have to be replaced using fertilisers. As noted above, organic management practices only allow FYM and several rock based fertilisers, such as rock phosphate, chalk and sylvite (providing P, Ca and K, respectively) as agents to provide these minerals. Due to this, organic soils are often shown to have slow rates of decline in the levels of P and K (Watson et al., 2002, Trewavas, 2001). A significant number of organic soils showed deficits in available P and K; 86% had P deficiencies and 36% showed K deficiencies. In contrast, only 15% and 30% of conventional soils examined showed deficits in P and K, respectively (Goulding et al., 2000).

Soil structure is assumed to be strengthened with the addition of organic matter. However, a review of the literature seems to indicate that this is not the case. A few, slight differences were seen in the amount of organic matter between organic and conventional pasture. Fields under arable practices showed greater amounts of organic matter when organically farmed. The major differences were seen when the conventional farm was one under stockless management; a practice that is not seen often in organic farms (Shepherd et al., 2002). However, this review, and the literature within, often does not take into account the correct matching of management, to allow an accurate conclusion to be drawn. Many conventional and Integrated Farm Management (IFM) farms will use FYM on their fields, so any differences seen between a stockless conventional farm and an organic farm would likely not be seen in a comparison where IFM is used.

In conclusion it appears that with the increase of IFM and best practice conventional farming in the UK, many of the supposed benefits of organic agriculture to soil quality and fertility are no longer true.

1. 3. Microbial community analysis of soils in organic and conventional agriculture

The various methods of Microbial Community Analysis (MCA) allow identification of bacterial genera and species that cannot be cultured in the laboratory (Head et al., 1998). PCR of ribosomal RNA and DNA, followed with Denaturing Gradient Gel Electrophoresis (DGGE) can be used to identify the bacterial diversity within a soil sample (Muyzer et al., 1993; Griffiths et al., 2000; Jenkins et al., 2010).

With the introduction of reliable DNA sequencers, a new method for analysis of the microbial community has been introduced. This method is called Terminal-Restriction Fragment Length Polymorphisms (T-RFLP). This method is similar to standard RFLP (Restriction Fragment Length Polymorphisms) analysis but a fluorescently labelled primer is used to amplify the rRNA or rDNA from an environmental sample and the product is digested with restriction enzymes. A DNA sequencer can then be used to identify the length of each fluorescently labelled restriction fragment (Liu et al., 1997). This technique, when coupled with online databases, such as the Ribosomal Database Project (Cole et al., 2005) can be used to identify genera present within a sample, based on terminal RF length. Statistical analysis, such as principle component analysis (PCA) and multidimensional scaling (MDS), are used to show changes in community structure over various parameters (Terahara et al., 2009).

The soil community is able to respond to the addition of soil amendments. Perez-Piqueres et al. (2006) showed large community changes in soil amended with spent mushroom compost (MC) when compared to the unamended soil. They suggest that this change is likely to be caused by the addition of the MC from the compost, with some stimulation of the original soil community. This trend holds true for both the bacterial and fungal communities. FYM and conventional treatments also affect the TRFLP profile of soils (Wolsing and Priemé, 2004; Widmer et al., 2006). While most T-RFLP studies carried out to date are based on 16S rRNA genes, Wolsing & Priemé (2004) used two nitrite reductase genes: nirK and nirS which allowed a certain level of targeting to denitrifying bacteria. This study, while not including replicate amendment treatments, did show that the three amendment techniques highlighted statistically significant diverse groups of bacteria. This trend was seen with both genes used for T-RFLP analysis, but not for all the restriction enzymes used for digestion. With nirS, two of the restriction enzymes showed disperse groups, while the third did not. This was not seen with nirK where all three restriction enzymes produced disperse groups. Furthermore, the nirK samples also showed a distinct change in microbial community over time. According to the authors, this was to be expected, as it has been seen in other studies using different techniques, such as the Phospholipid Fatty Acid analysis method (PLFA) (Bossio et al., 1998).

Widmer et al. (2006) used the 16S rRNA method on soil from a long running trial in Switzerland (the DOK field trial, established in 1978). This trial was set up to compare "biodynamic", organic and conventional management practices. The conventional management was sub-divided into conventional with FYM amendment and conventional with chemical fertilliser application (Mäder et al., 2002). The methods used by Widmer et al. showed significant differences between the FYM treatments when compared to plots with mineral or no amendment. The FYM plots had higher biomass and levels of extractable DNA. Community Level Substrate Utilisation and T-RFLP methods allowed separation of each regime by cluster analysis (Figures 1.5 and 1.6) (Widmer et al., 2006).

The T-RFLP derived diagram shows clear separation between mineral and FYM amendments and a separation between the two cover crops in FYM plots; Grass Clover and Winter Wheat (Figure 1.5). A similar pattern is seen with the CLSU cladogram (Figure 1.6). However, the non-amended plots do not cluster as closely to the mineral amended plots as in the T-RFLP cladogram. Again, there is a separation of FYM plots with different cover crops. However, the differences between biodynamic and organic treatments are small and not significant. This has also been noted in a short term trial of biodynamic treatments when compared to organic amendments (Carpenter-Boggs et al., 2000).

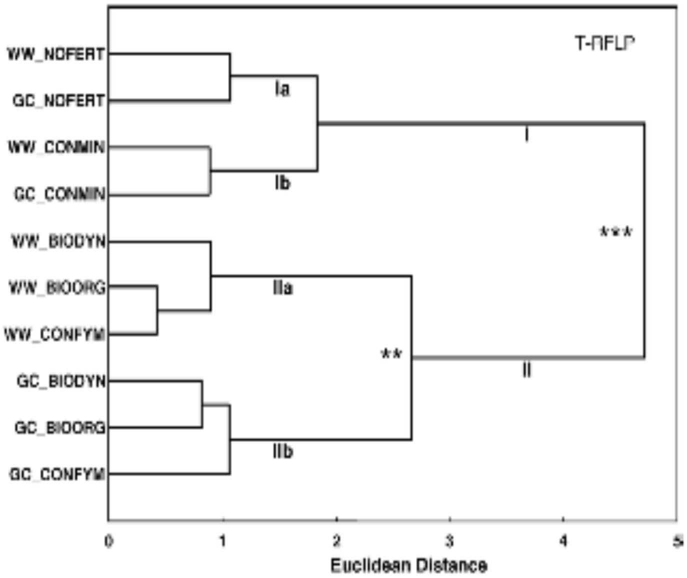

Figure 1.5: Cluster Analysis of T-RFLP profiles from Widmer et al (2006). BIOORG - bio-organic, BIODYN - bio-dynamic, CONFYM - conventional, CONMIN - conventional with mineral fertiliser NOFERT - No treatment, GC - Grass Clover, WW - Winter Wheat

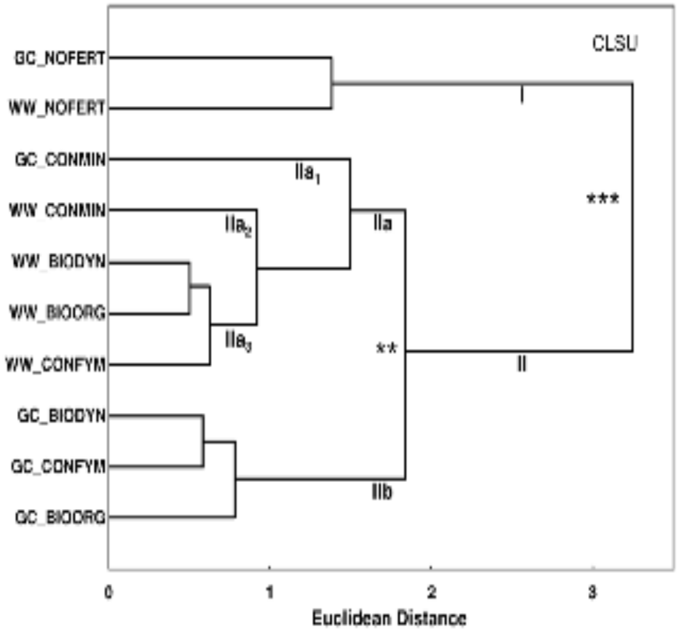

Figure 1.6: Cluster Analysis of CLSU data from Widmer et al (2006). BIOORG - bio-organic, BIODYN - bio-dynamic, CONFYM - conventional, CONMIN - conventional with mineral fertiliser NOFERT - No treatment, GC - Grass Clover, WW - Winter Wheat

The effect of amendment on soil bacterial profiles is also shown in other research. Bossio et al. (1998) showed distinct Phospholipid Fatty Acid profiles for low-input, organic and conventional management systems. However, this study implies that management practices are less important for altering microbial diversity with soil type, time and the specific mode of farming operation appearing to be more important. With respect to the study by Widmer and his other colleagues, this appears to hold true. Cover crop, part of the specific mode of farming operation, caused grouping of the FYM treatments from the T-RFLP and CSLU results.

1. 4. Compost amendments and disease suppression

Since the 1970s, composted amendments have been added to soils to add extra fertility and also to suppress fungal and bacterial diseases (Zhang et al., 1996). It is believed that the amendments induce Systemic Acquired Resistance (SAR) within the plant. SAR is induced by either a biotic or an abiotic elicitor and the threat to the plant is transmitted to undamaged areas by salicylate, which causes production of Pathogenesis-Related Proteins (PR) (Figure 1.7) (Vallad and Goodman, 2004).

It has been established that composted materials contain a microbial community associated with suppression of disease. In a suppressive decomposed peat mix, *Pseudomonas* species dominated, while in a conducive peat mix the dominant taxa were *Arthobacter* and *Bacillus* species (Boehm et al., 1993). Interestingly, it was a less decomposed mix that was more suppressive than a totally composted mix. Bacterial strains were isolated from the root tips and a higher percentage of bacteria from the suppressive composts were more effective at preventing *Pythium* damp-off in cucumber than ones isolated from the conducive composts.

Further study has focussed mainly on how compost affects plants. Zhang et al. (1996) showed that pine and spruce bark based compost could reduce the severity of *Phythium* root rot in cucumber when compared to a peat mix, which led to an increase in root dry weight. Spruce bark compost significantly reduced anthracnose of the second leaf of cucumber germinated on the compost mix.

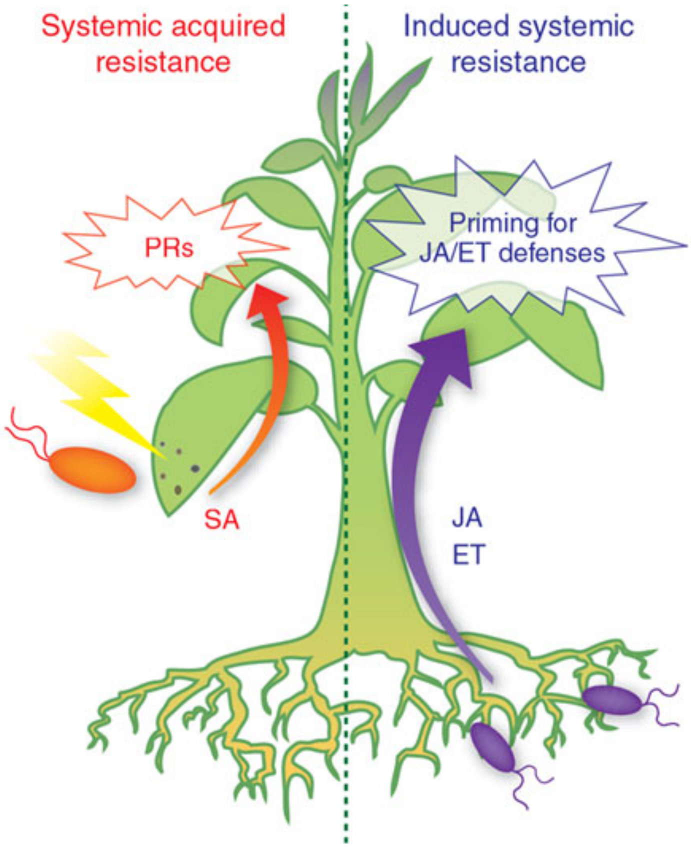

Figure 1.7: Diagrammatic representation of Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR). Salicylic acid is an essential signal molecule for the onset of SAR, as it is required for the activation of a large set of genes that encode pathogenesis-related proteins (PRs) with antimicrobial properties. ISR is typically activated upon colonization of roots by beneficial microorganisms. ISR is usually regulated by jasmonic acid or ethylene-dependent signaling pathways.

One of the markers for SAR used in the study was peroxidase activity (Zhang et al. 1996). Plants germinated in the compost mixes showed higher levels of perioxidase activity than those germinated in the peat mixes. Composts that had been inoculated with *Colletotrichum orbiculare*, a bacterial species that causes anthracnose, dramatically increased peroxidase activity in both the peat mix and the compost mixes. However, the response in the compost mixes was much higher than the peat mix indicating that the plant was already showing acquired resistance (Zhang et al. 1996).

In *Arabidopsis thaliana*, bacterial speck caused by *Pseudomonas syringae* pv. *maculicola* was reduced by composted pine bark (Zhang et al., 1998). Upon autoclaving, the suppressiveness was removed, but was restored on the re-application of compost material. This indicates that the nature of the agent causing SAR is a soil based microbial agent. Similar results were reported by Boehm et al (1993), who showed there were several bacterial species that induced SAR when applied to plant roots.

Monitoring the activity of β-1,3-glucanase in cucumber showed a similar pattern of expression as for peroxidase (Zhang et al. 1996). Levels were low in compost and peat mix, albeit higher in the compost mix. On inoculation with *C.orbiculare* the plants grown on the compost mix again showed significantly higher levels of activity compared with the peat mix plants (Weymann et al., 1995). In *Arabidopsis* GUS (β-D-glucouronidase), a reporter gene, was coupled to the promoter for PR2 (β-1,3-Glucanase). Again, a similar pattern of activity was seen as greatly increased levels of GUS activity was seen on induction with *C.orbiculare* in plants grown on compost mix. This lead the authors to the conclusion that SAR induced by compost amendments differed in mechanism of action from that induced by pathogens alone. In a classic SAR model, levels of β-1,3-Glucanase is rapidly increased to high levels, while in compost induced SAR enzyme levels are only increased after infection with the pathogen (Weymann et al., 1995).

Other sources of compost, such as cannery waste (from tomato processing waste, with added manure), also appears to induce SAR in tomato, albeit inconsistent over growing seasons (Abbasi et al., 2002). Both composted cannery waste and composted garden waste showed some protection against anthracnose and bacterial spot caused by *Xanthomonas axonopodis* pv. *vesicatoria.* However, while examining the progress of foliar diseases, it appeared that the compost amendments actually increased disease symptoms over time when compared with tomatoes grown in non-amended plots. In comparison, BTH (benzo (1,2,3) thiadiazole-7-carbothioic acid S-methyl ester), a common, commercial chemical elicitor slowed disease progress by over 50%. Interestingly, this study indicates that organic amendments also increased yields, something which is commented on elsewhere in this introduction (Abbasi et al., 2002).

Composted paper mill residue, a mixture of wood fibre fines, clay and minerals obtained from settling of wastewater, along with microbial biomass and effluent produced from an aeration process also acts to suppress disease. Over a two year trial in snap bean, four different treatments derived from paper mill residues all showed significant reduction of both snap bean *Pythium* damp-off and common root rot. When the residue was composted with bark, common rot root was reduced in the second year of the study without the need for further amendment (Stone et al., 2003).

At a gene expression level, there is further evidence that the composted amendment in some way initiates SAR (Vallad et al., 2003). In a growth chamber experiment using *Arabidopsis thaliana,* bacterial spot (caused by *Pseudomonas syringae* pv. *tomato*) was reduced, as has been seen before. Examination of the gene expression in several markers of SAR showed increased levels in plants grown in composted paper mill residue.

Mutants containing knockouts of jasmonate and ethylene response genes which are involved in the proposed Induced Systemic Resistance pathways (ISR) showed the same response as normal plants indicating that jasmonate and ethylene induction are not involved. However, NPR-1 Mutants showed increased severity of disease indicating that the pathogenesis related gene was involved. Levels of RNA expression showed a similar pattern; genes involved in classical SAR showed increased expression while those involved in ISR pathways showed no increases in expression.

Overall, the use of composted amendments does have a significant effect on providing additional, non-pesticide related defences to commercial crops. The soil environment appears to have added importance beyond sustaining crop growth (Vallad and Goodman, 2004).

1. 5. Plant response to organic and inorganic fertiliser

1. 5. (1) Differential expression at the transcriptomic and proteomic levels

Only one study, to date, appears to have been published investigating the effects of organic or inorganic fertilizer on the differential gene expression of wheat (Lu et al., 2005). This study used microarrays generated from a wheat grain endosperm Expressed Sequence Tags (ESTs) library to identify genes that were up-regulated in response to changing nitrogen levels. Twenty three genes involved in nitrogen metabolism were identified and shown to exhibit differential expression in plants amended with FYM. Of these 23, three were down-regulated when compared to plants with no nitrogen input. The other 20 were up-regulated (up to 5 fold) compared to no nitrogen input plants (Figure 1.8).

Microarray studies of *Arabidopsis thaliana* response to nitrate have shown that several genes with homology to those in wheat are also up-regulated (Wang et al., 2000; Wang et al., 2003). These genes include ones involved in nitrogen metabolism, as well as the gene encoding phosphoglycerate mutase. The enzyme product of this latter gene appears to have a role in either controlling nitrogen metabolism, or is actively involved in it. Both Wang et al. (2003) and Bahrman et al. (2004) found that this gene was up-regulated in response to increased nitrate levels. Bahrman et al. identified the gene at the proteomic level in wheat, while Wang et al. identified the gene at a transcriptional level in *A.thaliana*. Other genes, such as those encoding malate dehydrogenase, are also shown to be up-regulated by nitrate expression. However, this gene was not shown to be up-regulated in the work reported by Lu et al. (2005).

Nitrate transporters are one class of genes that are consistently up-regulated (Wang et al., 2000, Wang et al., 2003, Bahrman et al., 2004, Lu et al., 2005). Under FYM manure conditions, both the low affinity and high affinity nitrate transporters are up-regulated. As expected, these genes are also highly up-regulated in both *A.thaliana* and tomato.

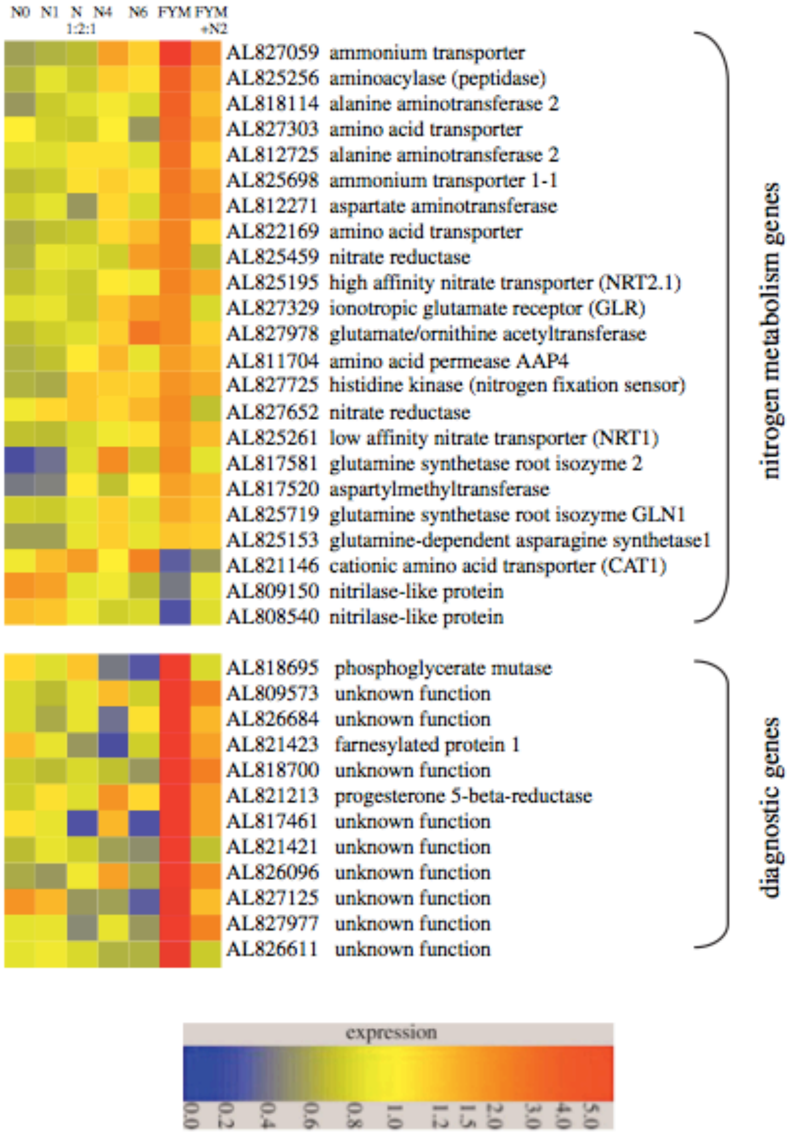

Figure 1.8: Transcriptional profiling showing differential regulation patterns of wheat genes when grown under different amendment regimes. (From Lu et al, 2005)

The study by Lu et al. also included 14 genes, which showed a consistent up-regulation in response to FYM. Several of these genes have no described function within the wheat genome and so were described as genes that could be used diagnostically to identify organically grown crops.

Recently, a study has been published focusing on the affects of management practices on the proteome of the potato tuber (Lehesranta et al., 2007). This study introduced crop protection management practices and crop rotation along with amendments regime as independent variables. PCA analysis of the results showed that only amendment regime affected the protein expression pattern.

One hundred and sixty protein spots showed significant differences between the two amendment regimes. One hundred and forty three were up-regulated in tubers grown under organic amendment while the remainder were up-regulated in conventionally grown potatoes. A significant number of the proteins up-regulated under organic conditions were identified as stress related proteins, such as member proteins of the 20S proteasome and Heat Shock Proteins (HSPs). Other proteins identified as up-regulated in stress response include superoxide dismutases (Bowler et al., 1992), Kunitz-type enzyme inhibitors (Koiwa et al., 1997) and proteins similar to β-cyanoalanine synthase, which is implicated in the detoxification of cyanide produced in tissues, possibly from the break-down of glucosinolates (Maruyama et al., 2001). Under conventional conditions, a small HSP and a proteinase inhibitor were more abundant. There also appeared to be higher levels of degradation products of patatin, a storage protein. The authors identify the increase of stress related proteins under organic conditions as supporting the hypothesis that organic crops grow under increased stress conditions due to insufficient nutrients (Zörb et al., 2006). However, as noted in section 6, these authors did not find any differences in the metabolome under organic conditions when compared to conventional conditions, including stress markers such as trehalose. There is also evidence that compost type amendments can cause the activation of systemic resistance pathways, which could explain the presence of upregulated related proteins in the organically treated plants (Vallad and Goodman, 2004). This point is also considered in section 4.

1. 5. (2) Physiological changes in response to management practices

Agricultural and agronomic studies on the physiology of plants grown under different soil management practices focus mainly on the yields produced by the systems under study. As noted earlier several long term field trials were designed to examine the differences between organic and conventional agricultural practices. Within each of these field trials, yield is one of the major descriptive outputs due to its agronomic significance.

The Broadbalk and Hoosfield long term field trials are possibly two of the most important of their type, due to the continuous collection of data since the trials were set up in the 19th century. Figure 1.4 shows the changes in wheat yield on the Broadbalk site since the trial was established in 1843. Yields from the continuous cropping plots have been similar over the course of the trial for both FYM application and the third level of nitrogen amendment. Yields of wheat in the first year after 2 years of non-wheat rotation are significantly higher than the continuously grown plots; this indicates that good rotation management practices are an important part of yield increases (Rothamsted Research, 2006). Until the 1980s, yields of spring barley grown with FYM matched those produced with conventional amendment on the Hoosfield field trial. However, more recently, yields of the long term FYM amended plots have become significantly higher than yields from plots with normal amendment (Figure 1.9) (Rothamsted Research, 2006).

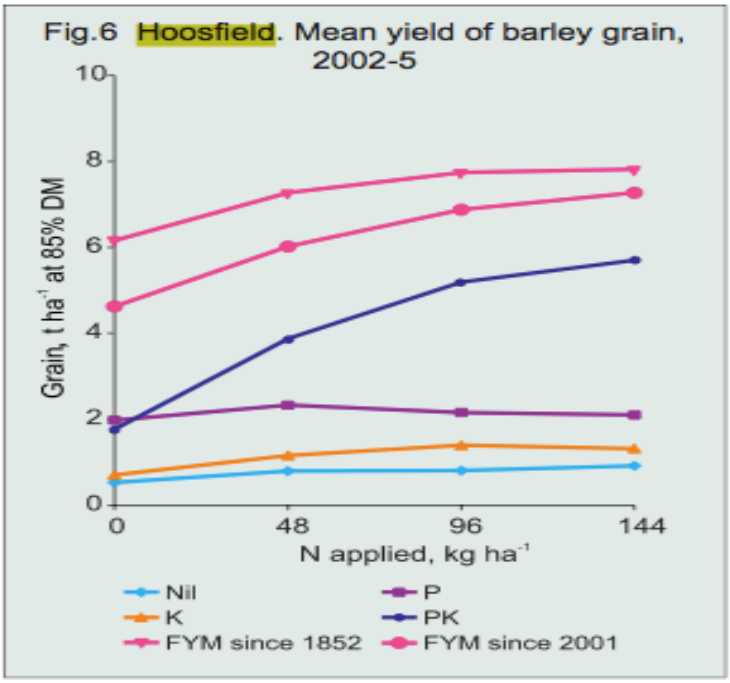

Figure 1.9: Hoosfield spring barley mean yields for the period 2002-2005. Different yields were obtained as a result of different fertilizer amendments (From Rothamsted Research 2006).

Other studies in the UK show significant reductions in yield when crops are grown organically. Leake, in a trial for CWS Agriculture (the farming division of the Co-Operative Society) showed significant decreases in the yield of several crops grown organically when compared to a conventional standard. Yields of wheat and oats where 68% and 81% of the coventional standard respectively.

However, while these drops in yield represent relatively small reductions, the author notes that there is a much larger reduction over the course of the entire rotation, as organic systems cannot sustain as many wheat crops as a conventional system. This is attributed to insufficient fertility and the lack of crops during ley stages of the rotation (Leake, 1999).

1. 6. Primary and secondary metabolism

Several reviews have been published on the role of organic agriculture in the production of secondary metabolites and in general support similar findings. While there are some systematic differences between the two management practices, most of the observed differences are inconsistent and small (Brandt and Molgaard, 2001, Woese et al., 1997). Only Vitamin C and nitrate levels are identified as having increased and decreased levels in organically grown crops, respectively (Woese et al., 1997).

Recently, a study has been published examining the levels of primary metabolites in wheat grain grown in the DOK trial in Switzerland (Zörb et al., 2006). This experiment used GC-MS to quantify levels of various primary metabolites present in the grain of wheat grown on the soil management systems set out in this trial i.e. conventional, organic and biodynamic practices. Interestingly, of the 52 metabolites positively identified, only eight showed any differences between treatments. These were two amino acids (alanine and valine), one sugar (myo-inositol), two organic acids (glycerate ad hydroxyglutarate), one vitamin and its precursor (panthothenic acid and β-alanine respectively) and urea.

The significant differences in these eight metabolites over the treatments are shown in Figure 1.10. The decreases between treatments were not consistent with all the metabolites. Panthothenic acid and β-alanine both decrease in the same order, with the no-input treatment having the highest levels and the biodynamic treatment being the lowest. This was, according to the authors, probably due to these two metabolites being in the same anabolic pathway. Valine and alanine were both expressed at higher levels in the conventional treatment, however, the authors explained that these levels were possibly being caused by the environmental conditions at the time of this experiment, as previous unpublished data had indicated no changes in the levels of these particular amino acids. The alterations of levels were also relatively small, so they would not have any effect on the nutritional value of the crop. Of the sugars and sugar alcohols identified, only one was up-regulated; myo-inositol. This was present in a higher level in the organic treatments. The differences in levels of all these metabolites, while statistically significant, is not large enough to impact directly upon human nutrition. There is currently no evidence that organically grown produce has any increased health benefits derived from non-nutrient food components, (Lotter, 2003; Magkos et al., 2006). However, some studies have been designed to examine the levels of various secondary metabolites. Håkkinen and Torronen (2000) investigated the levels of several phenolic acids and flavonols in strawberry (*Fragaria x ananassa*) cultivars when grown in different geographical locations and using different cultivation regimes. For the cultivation analysis, they expected to see a higher level of phenolic compounds in strawberries grown organically. This phenonmenon is due to an association of increased levels of biotic and abiotic stresses, which cause up-regulation of phenolic secondary metabolites with organic agriculture (Dixon and Paiva, 1995). However, this was not the case in the study by Håkkinen and Torronen where only one of six cultivars ("Jonsok") showed an increase in total phenolic compound concentration. This was attributed to increased levels of Kaempferol, an anti-microbial phenolic and was considered to be a general plant response to increased levels of pathogen attack, due to the lack of pesticides (Håkkinen and Torronen, 2000).

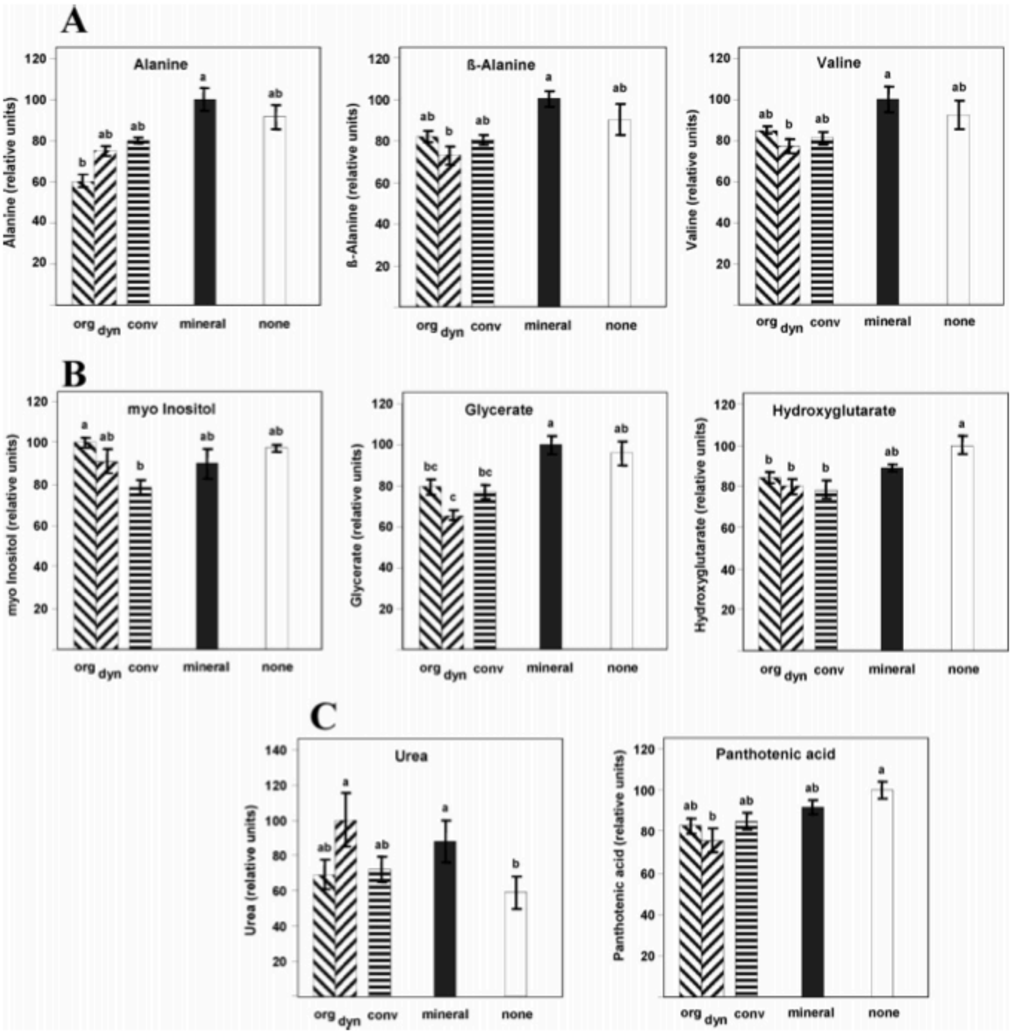

Figure 1.10: Metabolite content of wheat grains. A) Amino acids, B) Sugars and sugar alcohols C) Others. Different letters indicate statistical differences of the means. Org = organic, dyn = biodynamic, conv = conventional with FYM, mineral = conventional, none = no amendment (From Zörb et al 2006).

Conversely, Carbonaro et al. (2002) and Lombardi-Boccia et al. (2004) have shown that secondary metabolites are increased with organic management methods. Carbonaro et al. (2002) examined the levels of polyphenols in two common fruits: peach (*Prunus persica* L.) and pear (*Pyrus communis* L.). In both fruits, total levels of polyphenols were significantly increased with organic agriculture, a response which may be due to the lack of pesticides within this system. Insect predation is increased as they are not being killed by insecticide application, so there is more pressure on the plant to defend itself. They also show that polyphenoloxidase activity is increased in both organically grown peaches and pears. Polyphenoloxidase acts by oxidising phenolics so they form dark pigments, which help the plant repair the exposed surface. The toxicity of these pigments is also increased towards plant pathogens.

Lombardi-Boccia et al. (2004) studied the effect of three different methods of organic management on plum (*Prunus domestica* L.) when compared to conventional management. The three different types of organic management were based on cultivation techniques: tilled soil, soil covered with *Trifolium subterraneum* L. or soil covered with natural grass meadow. These three cultivation techniques caused significant differences in levels of both antioxidant vitamins and phenolics. Interestingly, the total polyphenol levels were higher in conventionally grown fruit, while phenolic acids, such as caffeic and chlorogenic acids, were higher in the tilled soil organic management technique. Quercetin, the most abundant flavonol overall, was also at higher levels in the conventionally grown fruit. Within the three different organic cultivation techniques, the trifolium cover management technique allowed higher levels of several phenolic acids and slightly raised levels of myricetin, a flavonol.

Other studies have also shown increases in the levels of products of secondary metabolism in plants grown with organic management methods when compared to conventional techniques. Caris-Veyrat et al., (2004) indicated that levels of the "microconstituents" examined were significantly higher on a fresh weight basis of organically grown tomatoes. In terms of dry matter, the effects were not as pronounced, but were still statistically significant, with the exception of β-carotene and naringenin, a flavonoid. Asami et al., (2003) reported that total phenolic levels were significantly higher in organically grown marionberries (*Rubus fructicosus*), "sustainably" grown strawberries (*Fragaria x ananassa*) and organically grown corn (*Zea mays*). However, this study came under heavy criticism due to several perceived problems with the experimental design. (Felsot and Rosen, 2004).

Other studies have examined the effects of plant derived secondary metabolites within the human diet. After a 22 day period where the food intake of the test group was either organically grown or conventionally grown fruit and vegetables, blood and urine levels of flavonoids were measured (Grinder-Pedersen et al., 2003). Higher levels of two of the major flavonoids (querecitin and kaempferol) were present in the diet and detected in the urine samples provided by the test subjects fed with organic produce. Markers of anti-oxidative activity, such as the TEAC (Trolox equivalent Antioxidant Capacity) and GR (glutathione reductase) levels were also examined. Interestingly, the organic diet decreased the TEAC levels, while the conventional diet increased them. According to the authors, this appeared to contradict the higher levels of flavonoids in the organic diet. However, they offer an explanation that this may be caused by other factors within the organically produced diet. GR levels were significantly increased by both diets over the time period indicating that the increased intake of antioxidant chemicals was affecting this. Organic agriculture often makes use of more pest resistant varieties to negate the need for pesticides. As one of the major plant defence mechanisms are the secondary metabolites, the differences in excreted flavonoids could have been caused by this varietal difference.

A similar study was performed by Caris-Veyrat et al. (2004) focused on one vegetable within the diet (tomatoes) and looked at plasma levels of two major antioxidants: ascorbic acid and lycopene, a carotenoid. While there was an increase in the plasma levels of these two chemicals during the trial, they were not statistically separated. The authors also considered that any effect of the organic tomatoes was obscured by the presence of conventional foods in the diets of the test subjects.

Antioxidants are also reported to have anti-mutagenic properties, due to their mode of action of removing active oxygen species which are capable of causing damage to DNA. One of the methods of testing chemicals with anti-mutagenic properties is a forward mutation test, where a bacterial species is grown with both the anti-mutagen and a potent mutagen. This experimental method has been applied to see if organically grown vegetables are better at lowering mutation rates than conventionally grown ones (Ren et al., 2001). Interestingly all of the vegetables selected for use in the trial were effective at reducing the levels of mutation caused by a specific mutagen. In the case of a few of the vegetables, the organically grown ones were significantly more effective in reducing the mutation levels than the conventionally grown ones. However, while the authors consider that this indicated that organically grown vegetables are "superior" to conventionally grown ones, there was no mention of varietal differences. As in the Grinder-Pedersen study, it is possible that any differences attributed to organic agricultural growing techniques could also be caused by choice of variety. Choice of pest resistant variety is a part of organic agriculture. However, when the hypothesis of an experiment involves linking soil management practice to a specific plant effect, variety needs to be removed as a variable.

Management practices that do not fit into the definition of either organic or conventional agriculture can also appear to have a profound effect on the levels of non-food constituents of crops. Wang et al. (2002) examined the phenolic and flavonoid content of strawberries grown with two different methods: hill plasticulture and matted row cultivation. Hill plasticulture grown strawberries had higher levels of many primary metabolites, such as sugars and ascorbic acid. Flavonoid and phenolic secondary metabolites were also higher in this cultivation technique. However, as these cultivation techniques were not identified as organic or conventional agriculture, this study will not feature further.

1. 7. Plant-insect interactions

There is a small body of literature on the effects of soil management on insect herbivory. This area encompasses two different viewpoints. One is that, while organic management does alter many variables, such as the increase of insect natural enemies, it does not actually affect the levels of damage when compared to conventionally managed farms (Drinkwater et al., 1995). The other viewpoint, proposed by Chaboussou, (1985) and Phelan et al. (1995) is that the organic amendment treatment causes a holistic response, so plants are healthier and better able to resist biotic stresses.

Letourneau et al. (1996) examined how soil management affects both the levels of nitrogen within the crop (tomato, *Lycopersicon esculentum* L.) and the amount of insect damage. Nitrogen levels within both shoots and leaves varied widely between both organically and conventionally managed farms. However, there was no positive correlation between these levels and the amount of insect damage from several different classes of herbivore. Field studies such as that carried out by Letourneau and Fox (1989) and Hanna et al. (1987) show a lack of a trend between tissue nitrogen levels and insect herbivory. However, when insect oviposition and herbivory levels are measured in small-scale field studies and glasshouse trials, there is a strong positive correlation between tissue nitrogen and insect damage, as well as other effects, such as fecundity (Scriber, 1984a; Scriber, 1984b; Letourneau and Fox, 1989; Minkenberg and Fredrix, 1989). Letourneau et al. (1996) give four possible explanations why, at a field scale level, tissue nitrogen levels and insect herbivory do not show the positive correlation that is shown in glasshouse studies. The response to higher nitrogen levels could be an insect species specific one; this study examined the cumulative insect damage which will only reflect population and community responses of many species.

Insect diversity, in both herbivores and natural enemies, is one factor that does change consistently with respect to organic and conventional farming practices. While there is no change in the abundance of phytophagous insects between conventional and organic tomato farms, there is a significant difference in arthropod biodiversity (Letourneau and Goldstein, 2001).

One of the major tenets of organic agriculture is that healthy soil produces healthy plants, which are not fed upon by insect pest species (Oelhaf, 1978). Phelan et al. (1995) attempted to put this claim into a scientific setting. In their study, soil from paired organic and conventional farms was used to grow Maize (*Zea mays*) and oviposition by the European Corn Borer (*Ostrinia nubilalis*; ECB) was measured. Statistical analysis of the results indicated that maize grown on conventionally treated soil had a higher level of oviposition than plants on the paired organic soils. Interestingly, amendment at the glasshouse level with different fertilisers had different effects. Generally, oviposition was lower on organic soils regardless of amendment, while different treatments affected the amount of oviposition with the conventional soils. However, there was no consistent effect here; of the three farm pairs tested, there was no significant interaction between soil type and amendment in one, while the remaining two farms showed different interactions. Furthermore, there was no significant interaction between plant biomass and oviposition.

Phelan et al. (1995) were able to draw the conclusion from this work that differences in the soil management practices were able to influence the ovipositional preference of ECB thereby indicating that mineral or nutrient differences in crops provide a non-genetic means of resistance to insect pests. Subsequently, Phelan et al. (1996), expanded upon this idea. They suggest that synthesis of final plant products occurs more readily when the plant's mineral balance is "optimal". When there is an "imbalance" of these minerals, the plants have a build up of precursor biochemicals, which can "provide an enriched diet for arthropod herbivores". These claims appear to be mainly supposition. While the results do appear to indicate that organically grown plants have a statistical balance of minerals, there was no evidence that this was directly linked to the observed lower levels of oviposition. There are several studies which appear to show effects similar to this. Alyokhin and his colleagues (Alyokhin and Atlihan, 2005; Alyokhin et al., 2005) studied Colorado Potato Beetle (CPB; *Leptinotarsa decemlineata* (Say)) and the effect of fertiliser amendment. Alyohkin et al (2005) investigated beetle damage related parameters, as well as plant physiological data over a five year field trial whereas the study by Alyokhin and Atlihan (2005) focused on beetle performance including mortality and female fecundity. The mean number of CPB life stages per plant over the course of the experiment is shown in Figure 1.10. The larval stages showed significant differences in at least three years of the experiment, as there was a much higher number per plant under the conventional amendment system. Under both systems, there was no significant difference in the amount of biomass and the canopy size of the plant. This suggested to the authors that these observed differences could not be linked to smaller plant size, or lower vigour.

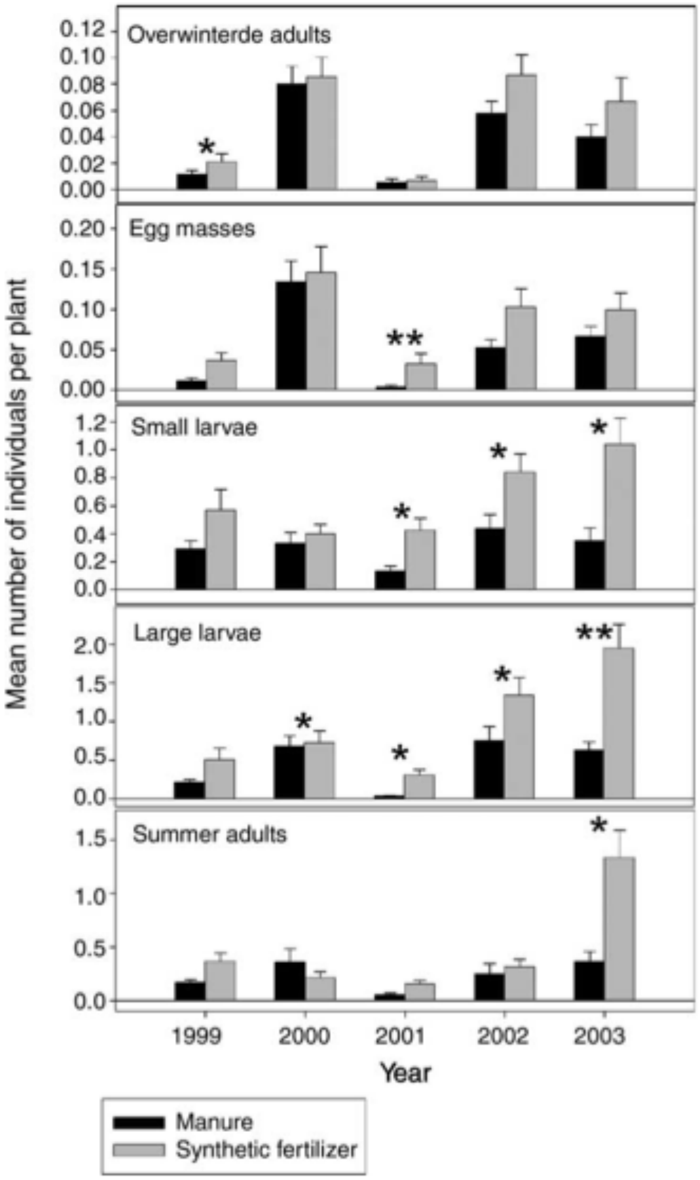

Figure 1.11: Levels of Colorado Potato Beetle infestation at various life stages on potato plants. significant differences: * P <0.05, ** P <0.01. (From Alyokhin et al, 2005)

Mineral analysis on the plants also showed significant effects similar to those seen by Phelan et al. (1995, 1996). Interestingly, boron is not mentioned by Phelan, but it has a large negative effect in this study. Concentrations of boron were two-fold higher in the manure-amended plants and all the CPB life stages, with the execption of overwintered adults which showed a negative effect to this mineral. Beanland et al. (2003) examined the effect of boron on insect feeding on soybean (*Glycine max*), under conditions of deficiency. The three insect pests used in this experiment, the soybean looper (*Pseudoplusia includens* (Walker)), the Mexican bean beetle (*Epilachna varivestis* (Mulsant)), and the velvetbean caterpillar (*Ancarsia gemmatalis* (Hübner)) all showed increased feeding on leaves from plants with no detectable boron, while plant growth was reduced in comparison to the control. The soybeans grown at the "optimal" mineral ratio performed much better, while the insect pests did worst on these plants. According to the authors, this is consistent with the mineral balance hypothesis.

The mineral balance hypothesis is based on an earlier comphrehensive theory by Francis Chaboussou (Chaboussou, 1985). In a book called "Healthy Crops, A New Agricultural Revolution", which has only recently been translated into English, the author lays out an argument that the application of synthetic chemicals to crops is the major cause for crop damage by insect damage. This theory is known as "trophobiosis". The relationship between plants and their predators is described as being primarily nutritive. Synthetic fertilisers cause the over-production of primary metabolites, such as sugars and free amino acids, which are of higher nutritional value to the insect predator. Pesticides act to alter the balance between protein breakdown and protein synthesis by causing an increase in the levels of protein breakdown further increasing the amounts of free amino acids present within the plant. By the removal of both synthetic fertilizer and pesticides, Chaboussou hypothesises that plants will be healthier and have lower pest pressure.

Overall, it appears that there is no clear consensus on whether organic management practices are able to prevent insect damage. There is evidence at both glasshouse and field trial levels that an "optimal" balance of nutrients within the plant will prevent insect predation. However, there is also evidence at field and farm levels that the use of organic farming techniques, while increasing insect diversity, do not affect pest numbers.

1. 8. Biodiversity within agricultural systems

It is considered that species richness within the agricultural ecosystem has declined dramatically over the last 50 years (Krebs et al., 1999, Tilman et al., 2002). The use of pesticides and mineral fertilisers has intensified at an individual field level, while the complexity of the agricultural landscape has decreased through the removal of semi-natural habitats to increase field size. It is a common belief that organic agriculture is better for the environment than other management techniques. For example, it is believed that species richness will be maintained at a higher level than conventional agriculture, due to the lack of pesticides and that complex landscapes should also support higher species richness (Roschewitz et al., 2005a). There are 5 main taxa usually studied in reference to agricultural biodiversity; microbes, plants, invertebrates, birds and small mammals. Microbial diversity is covered elsewhere in this introduction (see section 3).

In the Boarded Barns study (Aventis Science, 2000), a single farm based study that ran for 10 years in the UK, 80-85% of the biodiversity existed within field margins and hedgerows. This study compared Integrated Farm Management (IFM) practice (Drummond, 2000) with organic and conventional management practices. Many of the IFM landscape aspects are shared with organic practices, including extended field margins and hedgerows. The cropped areas of the Boarded Barns fields were of little significance in terms of biodiversity regardless of management practice (Higginbotham et al., 2000). Within this study, IFM no-till fields had a higher density of earthworms. Both Collembola and micro-arthropods were also higher in IFM fields, both in terms of individual numbers and species richness. Predatory arthropods, specifically the Carabidae, were more abundant on organic fields. However, the crop present in the field appeared to influence numbers more than the agricultural practice; bean crops had four-fold higher carabid numbers than wheat crops. For the carabids, when different farms are compared, there are similar increases in numbers and species richness on organic management (Clark, 1999, Bengtsson et al., 2005). However,while there were 3-fold higher numbers of beetles on the organic fields, Shah noted that *Pterostichus melanarius* was the most dominant beetle on the organic farms in their experimental plan, reducing α-diversity (Shah et al., 2003). However, other families of beetle, such as the Coccinellidae and Staphylinidae, were more common on conventional fields.

Declines in bird species on British farmlands has been well documented (Fuller et al., 1995, Whittingham, 2009). Some of the more general changes in agricultural management are believed to have directly affected farmland bird populations. The move from spring sowing to winter sowing that occurred during the 1970s has reduced the amount of winter stubble, a valuable seed source for birds during the winter. The lack of ploughing during spring in preparation for sowing has also removed sources of food for omnivorous species. Furthermore, a switch from rotations containing leys has probably limited niches available to bird species, as the landscape has become more homogenous.

Increased use of pesticides has also been considered as harmful to bird population numbers. This idea was first considered in “Silent Spring” (Carson, 1962). The use of wide-spectrum herbicides removed so-called weed species from the environment. These plant species provided an important seed source, leading to decline in bird numbers. A similar loss of insect abundance as a consequence of insecticide spraying will have affected insectivorous birds (Fuller et al., 1995, Krebs et al., 1999). However, species that are not traditionally farmland birds have also decreased, indicating that these declines in population cannot be explained simply by a change in agricultural management and loss of habitat. For example, the number of feral cats in the UK is believed to be over 1 million, while the number of domesticated cats is 8 million and has increased by 50% in the last 20 years. It is considered that these cat populations will have killed 300 million birds and small mammals every year, a significant loss of numbers (Trewavas, 2004).

The Boarded Barns study examined 13 different farmland bird species over the ten year duration of the experiment. There were intermittent higher levels of birds on organically amended fields in this experiment, but over the course of the entire ten years there were no significant differences between the two amendment regimes.

Chamberlain et al. (1999) studied bird populations on 22 matched organic and conventional farm pairs over a period of three years. Higher levels of birds were observed on organic farms compared with conventional farms. However, this increase of density was only shown to be statistically significant in one season for 8 of the 18 species identified. Increased overall density was seen during the winter months, with this higher density being on the organic farms. The authors posit that the birds are aligning according to resource availability. Overall, there was no statistical difference observed in species diversity. The authors believed that this increased density on organic farms was caused by them having larger and more heterogenous field margins which provided more habitats for nesting and greater levels of food sources. However, Integrated Farm Management (IFM) practices also include wide field margins and hedgerows (Drummond, 2000,Trewavas, 2004). The use of no-till agriculture, which is a major feature of IFM, increased the number of birds during the winter by an order of magnitude when compared to tilled fields (Higginbotham et al., 2000). Indeed, integration of "biodiversity conservation" measures, including field margins, increased bird population numbers by 42% in a period of 6 years on a conventionally managed farm (Boatman and Stoate, 2000).

Plant biodiversity refers to species that are usually considered as weeds, so in the cropping area a reduced number of species would be expected both in organic and conventionally grown arable crops. In the UK, the level of weed cover was not affected by the management practice, while the number of weed species was higher on organic farms than conventional ones (Moreby et al., 1994). However, grass species were not affected; percentage cover of both individual plants and number of species present showed no significant differences. Broad leafed species were more abundant and had greater percentage cover on organic farms. Three broad leafed species, *Tripleurospermum inodorum* Schultz Bip, *Stellaria media* (L.) and *Papaver rhoeas* L., showed significant abundance differences between fields and over both years of the study (Moreby et al., 1994). However, the authors sampled only within three metres of the field boundary in the headland of each field, an area which would likely receive no weed removal in organic fields and a minimal amount of herbicide treatment in conventional fields, leading to relatively high biodiversity in both management types.

Other British studies have shown a similar significant difference, even extending into the field. It can be seen from Figure 1.11 that consistently higher amounts of weed cover occurs in organic fields regardless of distance into the field, when compared to conventional fields (Fuller et al., 2005).

Other studies have also shown significant differences in plant biodiversity. A study in Sweden showed that the species diversity in both vegetation and the seed bank was higher in organic fields than in conventional ones. However, as landscape complexity increased (landscapes which were mainly arable fields are considered as simple, while landscapes containing a lower percentage of arable fields and areas of non-cropped habitats, such as field margins and grasslands, were considered complex), species diversity in conventional fields was increased. In the most complex landscapes, vegetation diversity was similar in fields from both management practices. Seed bank diversity was always increased by complexity in landscapes, regardless of management practices (Roschewitz et al., 2005a).

As highlighted by Hole et al. (2005), there are significant methodological issues to consider when comparing these studies. Due to country to country variation of organic standards, there is often no clear definition of what each country describes as 'organic'. The same applies to conventionally managed farms. For studies where more than one farm is used, there is often no information about how much the management practices differ between farms. There are also problems with matching farms due to landscape complexity. As noted by Roschewitz et al. (2005), the complexity of the landscape can significantly change the levels of biodiversity within a studied area. Roschewitz and his colleagues made a conscious effort to match the levels of landscape complexity to eliminate this source of error (Roschewitz et al., 2005a, Roschewitz et al., 2005b). Bengtsson et al. (2005) showed in their meta-analysis that plot and field scale studies often had positive effects from organic agriculture. Studies where landscape was matched showed no effects of management practice on plant biodiversity.

There is variation in the spatial scales used for studies of this type. For plant and invertebrate studies, field or plot scale tend to be used, while for vertebrates farm scale studies are carried out. Field scale studies will identify key management practices, but will lose any system-level effects (Hole et al., 2005). However, single farm and field/plot scale studies will reduce the kind of managerial differences mentioned above (Trewavas, 2004).

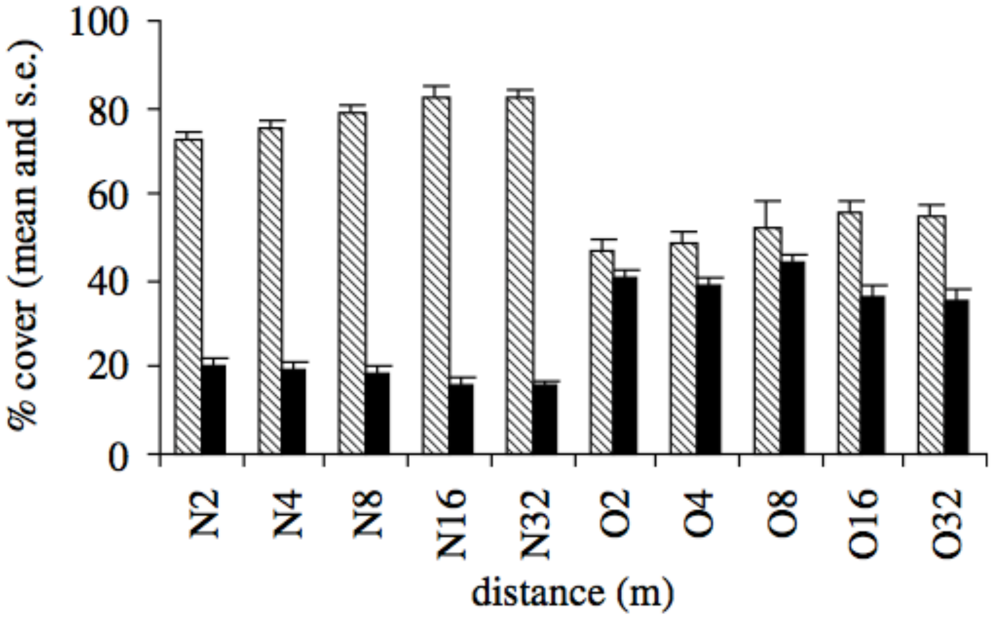

Figure 1.12: Crop and weed cover along transects into conventionally managed fields (N) and organically managed fields (O). Hatched bars indicate cover for cereal crop plants while black bars show weed cover values. Numbers are distances from the field edge. (From Fuller et al, 2005).

It appears that it is not a specific organic farming practice that increases biodiversity; it is more that elements which contribute to the complexity of the landscape help increase biodiversity. For example, wide field margins, hedgerows, mixed farming and classical rotations all add to the complexity of a landscape and also increase biodiversity levels (Chamberlain et al., 1999). However these elements are not exclusively the practice of organic agriculture; there is specific legislation in the UK to encourage farmers to set-aside land and increase the size of field margins, explicitly to increase biodiversity on agricultural land. These practices are also part of IFM (Drummond, 2000).

1. 9. Conclusions

Whether organic agriculture management practices have a role in creating a more sustainable agricultural system is still debatable. Many elements of organic farming are being integrated into conventional systems to provide benefits. The Integrated Farm Management programme is an example of this;

wider field margins have been introduced to increase biodiversity while crop rotations are expanded to reduce the chances of disease and add extra organic matter into the soil (Drummond, 2000).

It appears that organic agriculture does not produce anywhere near the yields of best-practice conventional agriculture indicating that organically produced food would always have a price premium, as increased availability leads to lower price for the consumer (Trewavas, 2004). Certainly it appears that elements that affect yield are likely to be increased on organic farms, such as insect pests and weeds. However, whether disease resistance is increased due to the presence of composts at a field level, as in the case of SAR for example, is un-studied.

At a plant molecular level, there is currently no evidence that organic agricultural practices have any positive effect on gene expression. In the studies carried out to date, several genes have been identified as being differentially regulated. In potatoes, for example, defence-related proteins are up-regulated indicating that the plant is under increased stress (Lehesranta et al., 2007). Again, whether increased stress has any advantage for the plant is debatable. Increased abiotic and biotic stresses, such as drought or insect herbivory, lead to lower yields, as the plant has to divert resources away from producing seed. However, at a metabolomic level, there is no evidence that the farming practice alters the levels of either primary or secondary metabolites, although the evidence for secondary metabolite levels changing is often extremely contradictory (Zörb et al., 2006, Brandt and Molgaard, 2001, Woese et al., 1997).

One of the major concerns drawn from the studies considered in this Introduction is that insufficient care has been taken to ensure that farms are matched correctly, as the type of farm will affect the results, as will the surrounding landscape. Because this often has not occurred, studies on a single farm are likely to provide a more accurate picture. Comparisons of yield on a trial such as the Broadbalk study are more useful than one where yields are compared between two separated farms.

Overall, it is management practice and how well this is controlled that affects all aspects of the farm. If the farm is managed badly, regardless of the type of practice used, biodiversity, yield and soil fertility will all suffer. However, a best practice conventional farm will show similar levels of biodiversity as an organic farm, and produce higher yields.

1. 10. Thesis rationale, aims and objectives

As noted above, very little is known about how the application of amendment agents, such as farmyard manure, to soil affects plants at a sub-cellular level.

The advent of post-genomic technologies enable the identification of target genes and their end products that are differentially expressed in plants in response to environmental perturbation, including soil fertility management practices; these changes may subsequently affect 'fitness' (productivity, stress tolerance).

The choice of Palace Leas as the source for all the soil used in this experiment was made for several reasons. First, it is a long-term experiment, with very infrequent changes made to the management of the site. This has led to a very consistent soil mineral profile, which is well documented. Two plots from Palace Leas were used throughout the experiments in this thesis, Plot 2, which is only amended using organic materials, and Plot 13, which receives inorganic nitrogen, phosphorous and potassium; these two plots thus represent contrasting fertilizer amendment regimes (Table 1.1). Pesticides have never been used on these plots.

The choice of *Arabidopsis thaliana* as the plant to be used throughout these experiments was a simple one. It remains the model organism for plant genomics, transcriptomics, proteomics and physiology. Currently, extensive annotated databases of genes and their protein products exist and are easily accessible.

The project seeks to identify the effects of different soil management practices (conventional/organic amendment) on microbial communities and the subsequent effects on the plant using *Arabidopsis thaliana* as a model species.

The project aim was investigated through the following objectives, each relating to specific chapters:

1) Identification of changes in the microbial community structure in response to conventional and organic management practice and amendment. Soils used were collected from the long term Palace Leas field trial where soil treatments have been documented for over 100 years (the trial was initiated in 1896). In this objective soil was taken from both Plot 2 (only amended using organic materials) and Plot 13 (which receives inorganic nitrogen, phosphorous and potassium); these two plots thus represent contrasting fertilizer amendment regimes. (See Chapter 2: Identification of the community structure of soil from the Palace Leas long term field trial using Terminal-Restriction Fragment Length Polymorphism technique)

2) Effects of soil management practice on plant growth and development. *Arabidopsis thaliana* was selected as the model plant system as its genetics are well understood and numerous ‘knockout’ mutants are available. Parameters measured included leaf number, plant size, seed yield, chlorophyll content, plant mineral analysis (See Chapter 3: Effects of soil management practice on plant growth)

3) Effects of soil management practice on plant gene expression at the proteome level. *Arabidopsis thaliana* was selected as the model plant system as its genome sequence has been fully annotated. Proteins were separated and identified by 2D-gel electrophoresis/MALDI-TOF MS (See Chapter 4: Effects of soil management practice on protein expression in *Arabidopsis thaliana* as a model plant)

4) Effects of soil management practice on plant volatiles and glucosinolates. Both direct and indirect chemical defences were studied in plants grown in soils representing the two different soil fertilizer amendment regimes. The subsequent consequences for insect herbivory were also investigated (See Chapter 5: volatile and glucosinolate production in *Arabidopsis thaliana* influenced by soil fertiliser amendement in response to insect herbivory).

5) Modelling the inter-relationships between soil microbial community structure (Chapter 2), plant growth and aphid fecundity (Chapter 3). Modelling was based on Structural Equation Modelling (See Chapter 6: The use of Structural Equation Modelling to identify interactions in a Soil-Plant-Aphid tritrophic system)

The experimental rational and design for the objectives are provided at the beginning of each chapter.

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Chapter 2. Elucidating the community structures of soils from the Palace Leas long term field trial using the Terminal-Restriction Fragment Length Polymorphism Technique

2. 1. Abstract

The microbial community is often a driver of plant growth and can respond to the addition of amendments to the soil. The aim of this study was to identify the community structure of both the bacterial and fungal soil communities and their response to time and additional fertiliser amendment. The soil came from two plots of the Palace Leas long-term field trial, Cockle Park Experimental Farm, Northumberland; Plot 2 which is amended with farmyard manure (FYM) and Plot 13 which is amended with inorganic (mineral) fertilisers. The changes in the community structure were measured using Terminal-Restriction Fragment Length Polymorphism. Each of the two soil types were amended with inorganic fertiliser or farmyard manure, or left unamended (i.e. 3 treatments for each plot). The microbial communities present in these soils were examined over time and the resulting data analysed using Detrended Correspondence Analysis. The laboratory addition of inorganic fertiliser had a significant effect on the bacterial community ($p=0.047$). However, neither initial field treatment practice nor time had any effect on the bacterial community ($p=0.263$, $p=0.842$, respectively). The fungal community showed no significant effect from field treatment, laboratory amendment or time ($p=0.342$, $p=0.131$, $p=0.219$). Changes in the structure of these communities is likely to be driven by another, unmeasured variable.

2. 2. Introduction

Ecosystems are highly dependent on the actions of micro-organisms. However, community structure and how it responds to environmental change has, in the past, been difficult to characterise (Bossio et al., 1998). This is due in part to the fact that the majority of prokaryote species present within the communities remain unidentified, a problem which precludes the use of traditional characterisation techniques. Several techniques are now available for determining community structures, all of which rely on individual species having subtle differences which can then be identified using appropriate procedures. Useful approaches include phospholipid fatty acid analysis (PLFA), which uses the difference in membrane lipids between species as an identification tool (Vestal and White, 1989). In addition there are several techniques based on DNA polymorphisms, often in rDNA genes such as the bacterial 16S rDNA gene (Head et al., 1998); these facilitate studies on population dynamics and time series.

Denaturing Gradient Gel Electrophoresis (DGGE) and Terminal Restriction Fragment Length Polymorphisms (T-RFLP) are based on PCR of a section of DNA followed by restriction enzyme digest. In DGGE, the fragments are separated on a denaturing acrylamide gel thereby allowing accurate sizing of fragments. In T-RFLP, one of the primers used for PCR is labelled with a fluorescent probe, which allows detection and sizing of fragments by DNA sequencing (Liu et al., 1997, Kim et al., 2010). The identification of the metagenome through these approaches, along with other methods such as shotgun Sanger sequencing and pyrosequencing, have allowed the identification of thousands of species present in microbial communities that were previously unknown (Handelsman et al., 1998, Eisen, 2007).

One of the main factors affecting the quantity of microbial biomass in any soil is the input of organic material into the system. This can introduce microbial species that were present in the organic material, as well as increasing levels of carbon (Shannon et al., 2002). Peacock et al (2001) showed that the application of farmyard manure (FYM) to soil increased C and N levels, along with microbial biomass (determined by PLFA) compared to control and samples amended with inorganic N. Community structure was also influenced by both FYM and the use of inorganic fertilisers, with an increase of PLFA markers associated with Gram-negative bacteria in the FYM treated soils, while addition of inorganic fertiliser caused a reduction in this group of bacteria. Other studies have shown significant increases in microbial parameters, such as biomass in plots under organic farming practices. Mäder et al (2002) showed significantly increased microbial biodiversity in the organic and biodynamic plots on the DOK (bioDynamic, bioOrganic and (K) conventional) long-term field trial in Switzerland. A corresponding decrease in the metabolic quotient ($q\text{CO}_2$)

indicated that these microbial communities were able to use the added organic material for growth rather than for maintenance and survival.

Other studies have confirmed these increased levels of microbial indicators in the DOK field trial (Widmer et al., 2006, Birkhofer et al., 2008). Both biomass and total extractable soil DNA showed significant, or almost significant, increases in plots amended with organic fertilisers, while colony forming unit measurements were influenced more by the cover crop than amendment regime. Substrate utilisation tests allowed separation of the amendment treatments by cluster analysis. However, Birkhofer et al. (2008) found there was evidence that soil carbon was more freely available to microorganisms in the conventional system.

In other trials, increases in microbial biomass, enzyme activity and other soil parameters were seen when organic fertililisers are used (Drinkwater et al., 1995, Bossio et al., 1998, Carpenter-Boggs et al., 2000, Bohme et al., 2005). However, while increases in biomass due to the application of FYM have been seen in other long-term field trials, there is conflicting evidence whether this actually stimulates increased levels of diversity within microbial communities. For example, Bossio et al. (1998) reported that the long-term field trial used in their studies showed increased microbial biomass in the organically treated plots, but these measurements were not associated with the PLFA profiles. There were changes to the PLFA profiles on addition of organic fertiliser, but groups of PLFA were both enriched and decreased in the organic plots. Community structure in this experiment were most affected by soil type, with management practice being somewhat less important. In the DOK field trial, cluster analysis was applied to the results generated by T-RFLP profiling. The main cause of community alteration was addition of FYM, whilst cover crop played a somewhat smaller role. The management system only showed a small but statistically insignificant effect on the community (Widmer et al., 2006).

Other studies have shown the effect of management practice and fertiliser amendment on the soil microbial communities (Marschner et al., 2003, Wolsing and Priemé, 2004). DGGE and redundancy statistical analysis showed significant strong differences between soils treated with manure, mineral fertiliser and straw at a low rate (Marschner et al., 2003). However, there was no obvious change in functional diversity, as shown by insignificant changes in enzyme activity. Use of PCR and T-RFLP targeted at nitrogen metabolism related genes revealed a strong response to the use of fertilisers. Nitrate reductase genes (*nirK* and *nirS*) were targeted and used to identify large community shifts due to the application of different fertilisers; however, differing levels of inorganic nitrogen input did not cause changes in the microbial community. With *nirK* there was also a significant response to seasonal change (Wolsing and Priemé, 2004).

Responses in the microbial community structures to compost and manure amendments have also been shown in soils from different regions of France (Edel-Hermann et al., 2004, Perez-Piqueres et al., 2006). In these studies fungal communities showed both regional and amendment differences (Edel-Hermann et al., 2004). The bacterial community was also altered by the addition of composts, but this change in community structure did not correlate with differences seen in the analysis of enzyme activity (Perez-Piqueres et al., 2006). However, it is likely that this is due to functional redundancy in the soil microbial community.

In the present study, the soil used came from Palace Leas hay meadow plots, the world's longest-running grazing and hay cutting experiment, initiated in 1896 at Newcastle University's Cockle Park Experimental Farm. The unreplicated plots have been amended with mineral fertilisers, FYM or a combination of the two since that time (Shiel, Personal Communication). These hay meadow plots have been well characterised at both the physical and biological levels (Arnold et al., 1976, Shiel and Hopkins, 1991, O’Donnell et al., 2001). Soil microbial biomass is higher in plots that receive FYM, and PLFA indicates a distinct separation in microbial communities between plots. Furthermore, discriminant analysis showed separation in microbial communities between the plots that receive FYM and those that receive inorganic fertiliser (O'Donnell et al., 2001).

The primary aim of this study was to identify the differences in microbial community structure between plots from the Palace Leas field trial that have received different fertiliser amendments. Soil from two plots were used: Plot 2, which is amended with farmyard manure (FYM) and Plot 13, which is amended with ammonium sulphate, sodium nitrate, triple superphosphate and potassium oxide. Soil from these plots were further amended with FYM or inorganic fertilisers after collection, to identify how these amendments cause changes in the community structure over time.

2. 3. Materials and methods

2. 3. (a) Soil types

The soil samples were taken from the Palace Leas long-term field trial at Cockle Park Experimental Farm, Newcastle University. The soil type is clay loam over clay (Hallsworth Series, pelo stagnogley (typic ochraqualf)). Soil was taken from Plots 2 and 13.. Plot 2 receives 20t ha^{-1} FYM annually, and is a straw-based farmyard manure produced from cattle reared at Cockle Park. Plot 13 receives 35kg ha^{-1} nitrogen in the form of ammonium sulphate and sodium nitrate, 60kg ha^{-1} phosphorus as triple superphosphate and 67kg ha^{-1} potassium as potassium nitrate.

2. 3. (b) Soil sampling

Cores were taken using an auger, with the sample being taken from a depth of 2-20cm. A total of 3kg of soil was taken from each plot. The soil samples were sieved to a diameter of 2mm to remove plant material. The sieved material was then stored at 4ºC until required.

2. 3. (c) Soil treatment

Three different amendments were applied to each plot in the laboratory; addition of FYM, addition of inorganic fertiliser and no additional treatment . Approximately 200g of soil were taken for each amendment. To add FYM, 20g was taken and mixed in by hand to the soil sample. Inorganic amendment was achieved by soaking the soil in a solution made up from the mineral fertilisers used at Cockle Park farm. A 10% solution of these compounds was prepared and then 200g of soil was placed in a plant pot and allowed to soak for 24 hours in this solution. Both the inorganic and un-amended soil had added perlite to allow for the extra mass of the organically amended plots. Once all the treatments were prepared, *Arabidopsis thaliana* (ecotype C24) were planted and the soils kept at 4ºC for 48 hours to allow vernalisation of the seeds. Each pot was placed in a growth room following a 16/8h light cycle and a 22ºC/20ºC temperature cycle.

2. 3. (d) Experimental sampling

At weekly intervals following the different treatments, 1g duplicate soil samples were taken over a period of 6 weeks. This is the length of time required to grow *Arabidopsis* to the size required for future experiments. All soil samples were frozen immediately at -80ºC until processed.

2. 3. (e) DNA extraction and purification

DNA from the soil samples were extracted using the method of Griffiths et al. (2000), but with a few modifications, specifically the second wash with chloroform-isoamyl alcohol to remove phenol contamination. All chemicals were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated. All solutions were prepared with DEPC treated water, while all plasticware were DNase and RNase-free.

0.5g (wet weight) soil was added to a 1.7ml screwcap centrifuge tube containing 0.5g glass beads. Extraction was performed by adding 0.5ml CTAB extraction buffer and 0.5ml phenol-chloroform-isoamyl alcohol (25:24:1 v/v, pH 8.0). The CTAB extraction buffers consisted of a 10% solution of hexadecyltrimethylammonium bromide in 0.7M sodium chloride mixed in equal parts with 240mM potassium phosphate buffer at pH 8.0. Samples were then lysed for 30s at a speed setting of 5.5ms^{-1} using a Hybald ribolyser, centrifuged at 13,000g for 5min and the aqueous phase was removed and retained. Any phenol contamination was removed by mixing the aqueous phase with chloroform-isoamyl alcohol (24:1) followed by centrifugation at 13,000g for 5 minutes. DNA was precipitated from the aqueous layer using 2 volumes of 30% polyethelene gycol 6000 in 1.6M NaCl. Samples were incubated for 2 hours at room temperature then centrifuged at 13,000g for 10 minutes. After removal of the supernatant, the samples were washed with ice cold 70% ethanol, followed by air-drying and re-suspension in 200µl Tris-EDTA buffer (10mM Tris, 1mM EDTA).

Due to high levels of humic acid contamination, an additional purification step adapted from Cullen and Hirsch, (1998) and Edel-Hermann et al., (2004) was required. Spin columns were made from 0.5ml microcentrifuge tubes, which had a small hole placed in the bottom of the tube. A small plug of glass wool was used to support the matrix. 100mg of of polyvinylpolypyrrolidone (PVPP) was added to each column. Two washes of DEPC-treated water were applied to bed down the PVPP matrix. The columns were prepared in advance and stored at 4ºC.

Samples were applied to the columns, allowed to drip into a collection tube for 5 minutes, then centrifuged at 1000g for 15 minutes at 10ºC. The flowthroughs were ethanol/sodium acetate precipitated, washed with 70% ethanol and the pellet re-suspended in 50µl of Tris-EDTA buffer. The extraction of DNA from each sample was confirmed using a 0.8% TAE-agarose gel and quantified using a Nanodrop spectrophotometer.

2. 3. (f) PCR and restriction enzyme digests

Two sets of PCR were run, one to amplify the bacterial 16S rDNA fragments and one to amplify the fungal SSU rDNA.

2. 3. (f) (i) 16S rDNA PCR

All amplifications were carried out in 30µl reaction mixtures, these contained 3µl 10x reaction buffer (1x contains 50mM Tris-HCl, 15mM $(NH_4)_2SO_4$, 0.1% Triton X-100) 2mM MgCl, 200µM of each dNTP, 2U of Dynazyme EXT DNA polymerase (Finnzymes, GRI, UK), 1pmol of each primer and 10ng of DNA. The primers are listed in Table 2.1. The conditions for the PCR were: 5 minutes at 94ºC, then 30 cycles of 30 seconds at 94ºC, 30 seconds at 56ºC and 30 seconds at 72ºC, followed by a final extension period of 10 minutes at 72ºC.

Table 2.1: Primer sequences used for PCR

Primer designation	Primer sequence	Reference
8f (bacterial)	AGA GTT TGA TCC TGG CTC AG	Amann et al., (1995)
926r (Bacterial)	CCG TCA ATT CCT TTR AGT TT	Muyzer et al., (1995)
FR1 (Fungal)	AIC CAT TCA ATC CGT AIT	Vainio and Hantula, (2000)
FF390 (fungal)	CGA TAA CGA ACG AGA CCT	Vainio and Hantula, (2000)

2. 3. (f) (ii) SSU rDNA PCR

Amplifications were performed in 30µl reaction measures, these contained 3µl 10x reaction buffer (1x contains 50mM Tris-HCl, 15mM $(NH_4)_2SO_4$, 1.5mM MgCl 0.1% Triton X-100), 20µM of each dNTP, 2U of DyNAzyme EXT DNA polymerase (Finnzymes, GRI, UK) 1pmol of each primer and 10ng of DNA. The primers are listed in Table 2.1. PCR conditions were 5 minutes at 94ºC, 30 cycles of 1 minute at 94ºC, 45 seconds at 50ºC and 2 minutes at 72ºC, followed by a final extension period of 10 minutes at 72ºC.

2. 3. (f) (iii) Restriction Enzyme Digestion

The bacterial samples were digested using the *Hha1* restriction enzyme (New England Biolabs, Massachusetts, USA). 15µl of PCR product was digested with 6U of *Hha1* for 16h at 37ºC, samples desalted using ethanol/sodium acetate and then resuspended in 7µl of molecular grade water. Fungal samples were digested *using HaeIII* restriction enzyme under the same conditions.

2. 3. (g) Fragment Profiling

2µl of each sample was mixed with 8µl of 0.1% v/v Tween-20 and 0.25µl of ET-900-R size marker. All of the fluorescently tagged fragments were profiled using a megaBACE sequencer (Molecular Dynamics, California, USA) in the genotyping mode. The fragment profile was viewed using Fragment Profiler Software (Molecular Dynamics, California, USA).

2. 3. (h) Statistical analyses

Statistical analyses was performed using the Canoco for Windows package (Plant Research International, Wageningen, Netherlands). Treatment, time and amendment were set as environmental variables, while fragment lengths were set as species data. Samples were subjected to Detrended Correspondence Analysis (DCA) with no transformation. Ordination plots were drawn using CanoDraw, part of the Canoco package. General Linear Model analysis was applied to each environmental variable to establish their significance as a driver of the system.

2. 4. Results

2. 4. (a) Verification of PCR Amplicons

PCR amplifications of bacterial 16S rDNA preparations were expected to give an amplicon of 918 base pairs. Agarose gel analysis of samples after PCR revealed the presence of a band at ~900bp in length, indicating successful PCR (Figure 2.1). PCR amplifications of the fungal SSU rDNA region was expected to give an amplicon of 390 base pairs in length. Again, the results of agarose gel analysis of the sample after PCR showed a band at ~400bp in length indicating successful PCR (Figure 2.2)

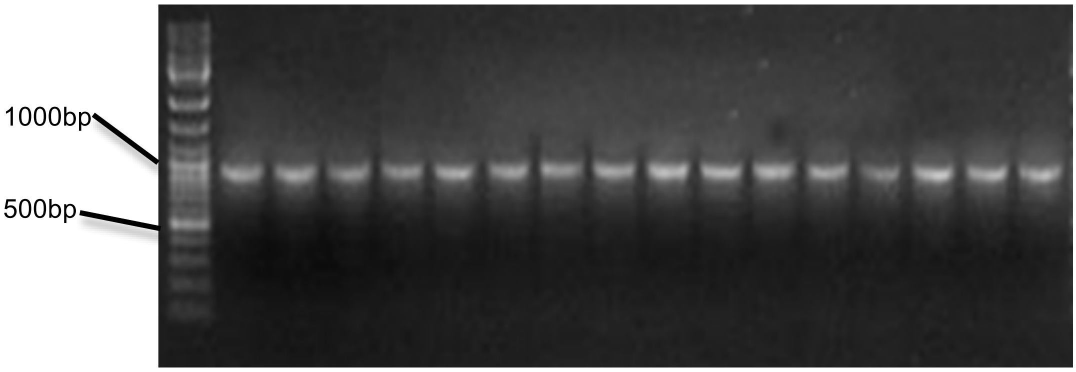

Figure 2.1: Agarose gel image of bacterial samples produced after PCR demonstrating the presence of a band at ~900bp in length, indicating successful amplification of 16S rDNA

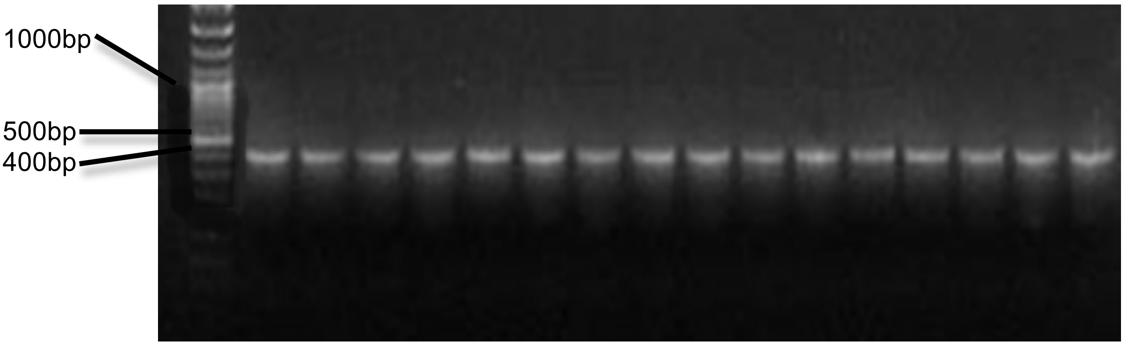

Figure 2.2: Agarose gel image of fungal samples produced after PCR demonstrating the presence of a band at ~400bp in length, indicating successful amplification of SSU rDNA

2. 4. (b) Statistical Analyses of the Bacterial Communities

The scores of the first two ordination axes were plotted for each sample (Figure 2.3) and for each bacterial “species” (length of each terminal restriction fragment) (Figure 2.4 A). These scores show that there is little separation between the two soil types regarding the bacterial community structure (tr_inorg=plot 13, tr_org=plot 2, Figure 2.3 A). ANOVA analysis of the first axis sample scores (using the General Linear method) showed that there was no statistical difference between these two treatments (p=0.263, F=1.27, Figure 2.5). This indicates that the original soil treatment regime has had no effect on the bacterial community structure.

When the ordination plot samples were attributed to laboratory amendment, a distinct grouping can be seen (Figure 2.3 B). Samples that received inorganic amendment form a cluster in the centre of the graph, while samples that received no laboratory amendment or organic amendment cluster together. ANOVA analysis of the first axis sample scores showed that there was significant statistical difference between the three amendments (p=0.047, F=3.18, Figure 2.5 A). Examination of the results of Tukey's ranked test showed that there was significant difference between the soils amended inorganically in the lab and those amended with FYM (p=0.0467, Figure 2.5 A)
The General Linear Method of ANOVA also allows interactions between variables to be analysed. In this case, there were no significant interactions between original soil treatment and laboratory amendment (p=0.604 F=0.51) in the bacterial community structure.

Time was analysed as a covariate in the ANOVA GLM method. Time showed no significance on the axis 1 DCA scores (p=0.842, F=0.04)

2. 4. (c). Statistical Analyses of the Fungal Communities

The DCA derived sample scores for the first two ordination axes were plotted (Figure 2.6), as were the "species" scores (Figure 2.4 B). There is some clustering of samples that were amended at the field level with FYM fertiliser (Figure 2.6A). However, analysis with ANOVA (general linear method) showed no statistical difference between the two field applied treatments (p=0.342, F=0.92). This indicates that the field application of fertilisers has no effect on the fungal community structure.

Identifying the ordination plot samples by laboratory amendment shows some clustering, particularly of samples that received FYM in the laboratory. Analysis with ANOVA revealed no significant difference between the three laboratory amendments (p=0.131, F=2.09), indicating that the laboratory amendment of samples has no effect on the fungal community structure (Figure 2.5B). ANOVA analysis of the interaction between field and laboratory amendment showed no significant difference (p=0.857, F=0.15, Figure 2.5B).

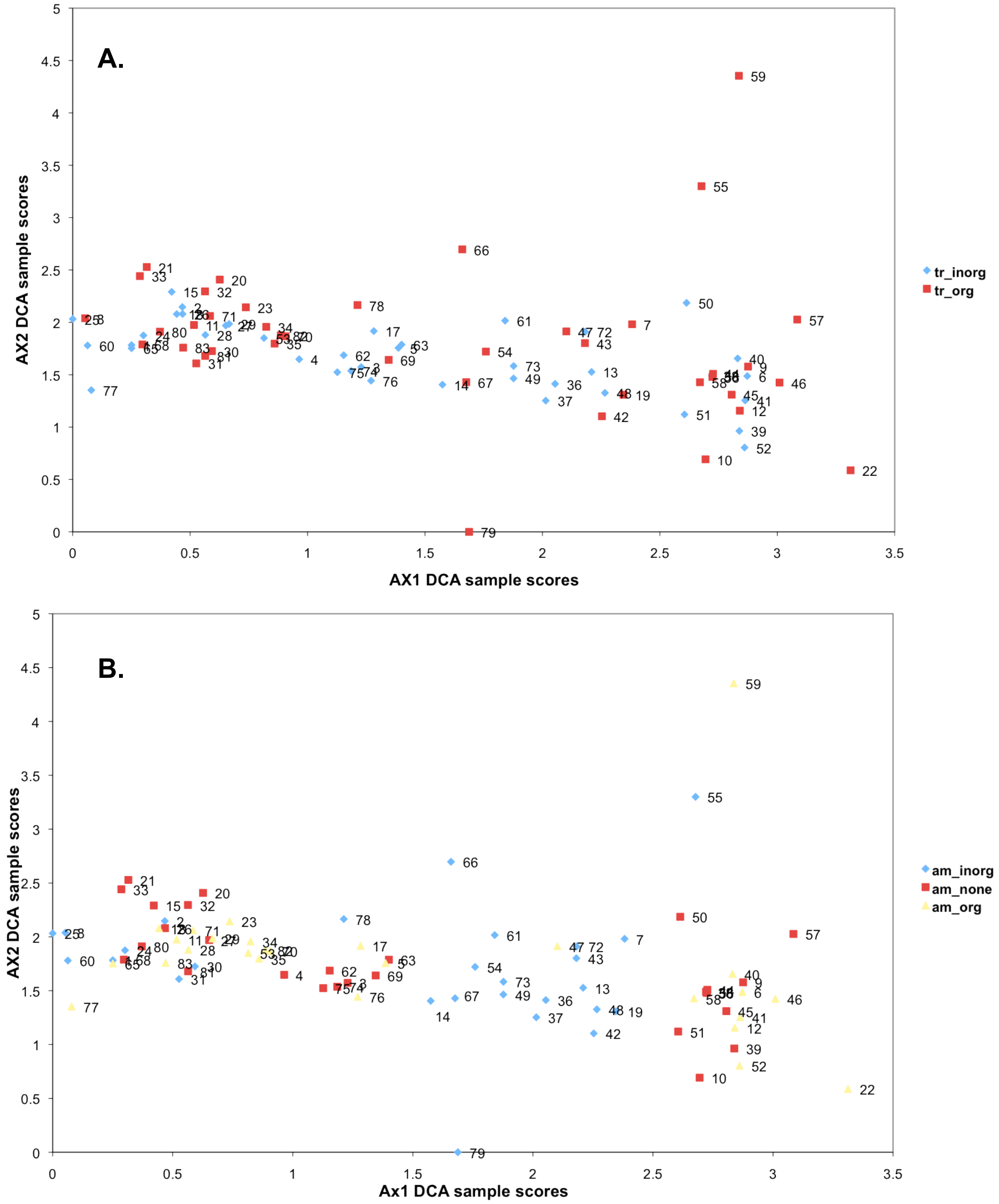

Figure 2.3: Bacterial community first axis sample ordination scores derived from DCA, plotted against second axis ordination scores. A) Samples grouped by original treatment (tr_inorg, mineral fertilizer amendment= Plot 13; tr_org, FYM amendment= Plot 2). B) Samples grouped by laboratory amendment (am_inorg= addition of mineral fertiliser, am_org= addition of FYM, Am_none= no further amendment)

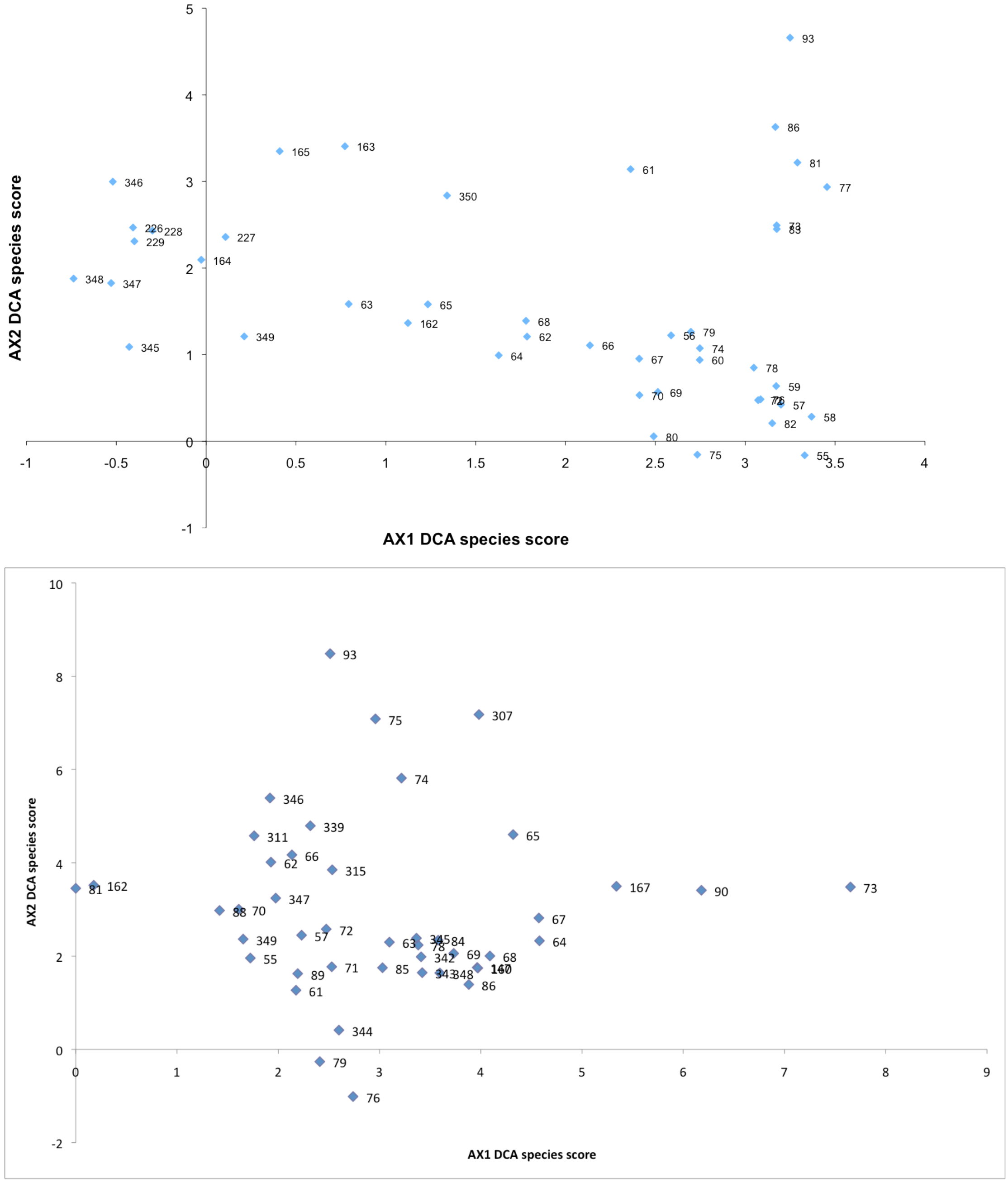

Figure 2.4: Species (length of terminal restriction fragment) first axis ordination scores plotted against second axis ordination score. A.) Subset of highest weighted (>50, as determined by DCA) species detected by the bacterial T-RFLP. B) Subset of the highest weighted (>20, as determined by DCA) species detected by the fungal T-RFLP

Time was analysed as a covariate within the ANOVA GLM. Again, time showed no significance, indicating that it has no effect as a factor on the DCA sample score results (p=0.219, F=1.54).

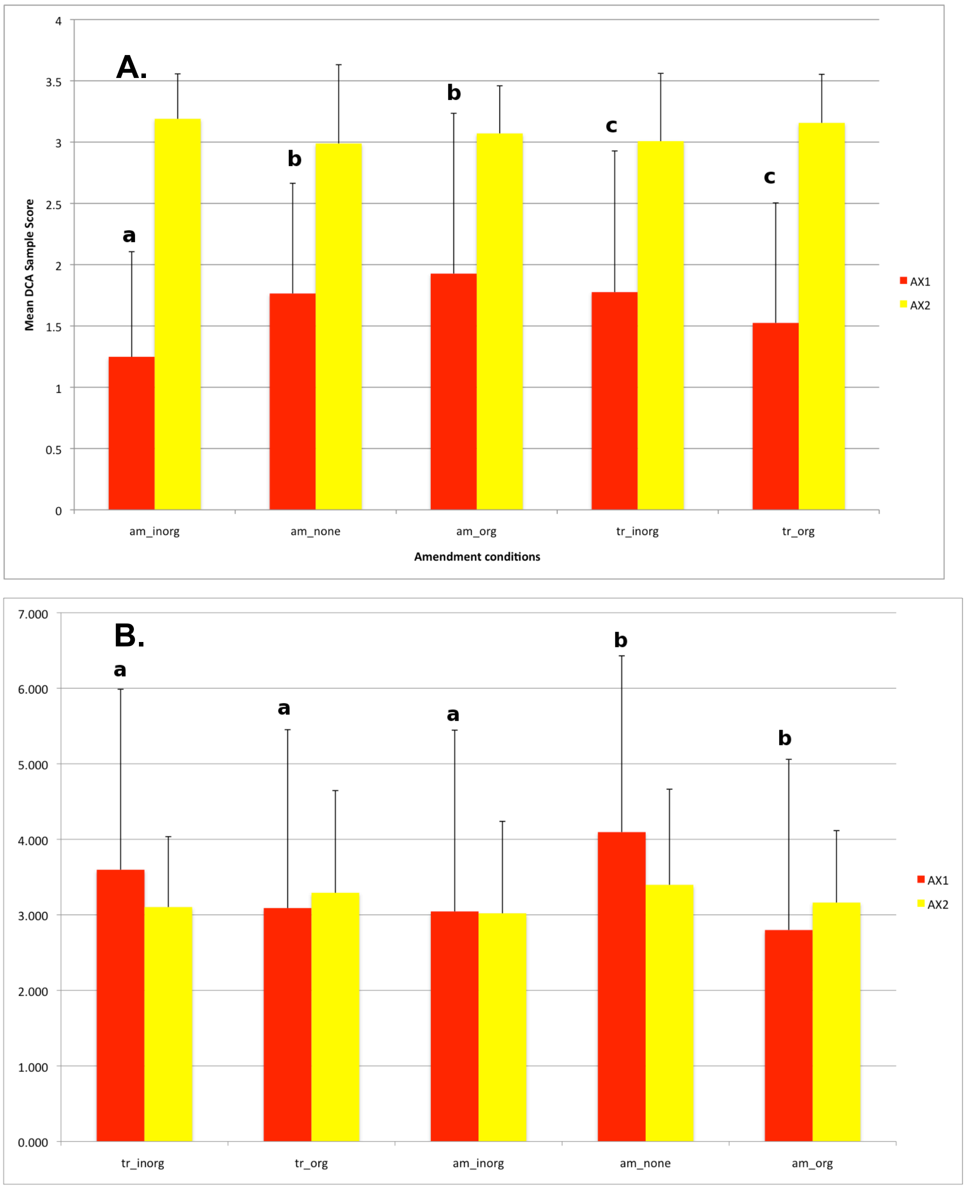

Figure 2.5: Plots of the mean DCA sample scores of both the first and second ordination axes for each field treatment and laboratory amendment. A) mean DCA sample scores from the bacterial T-RFLP. Lower case letters indicate significant differences between amendment type. B) Mean DCA sample scores from the fungal T-RFLP. Lower case letters indicate no significant differences between amendment type.

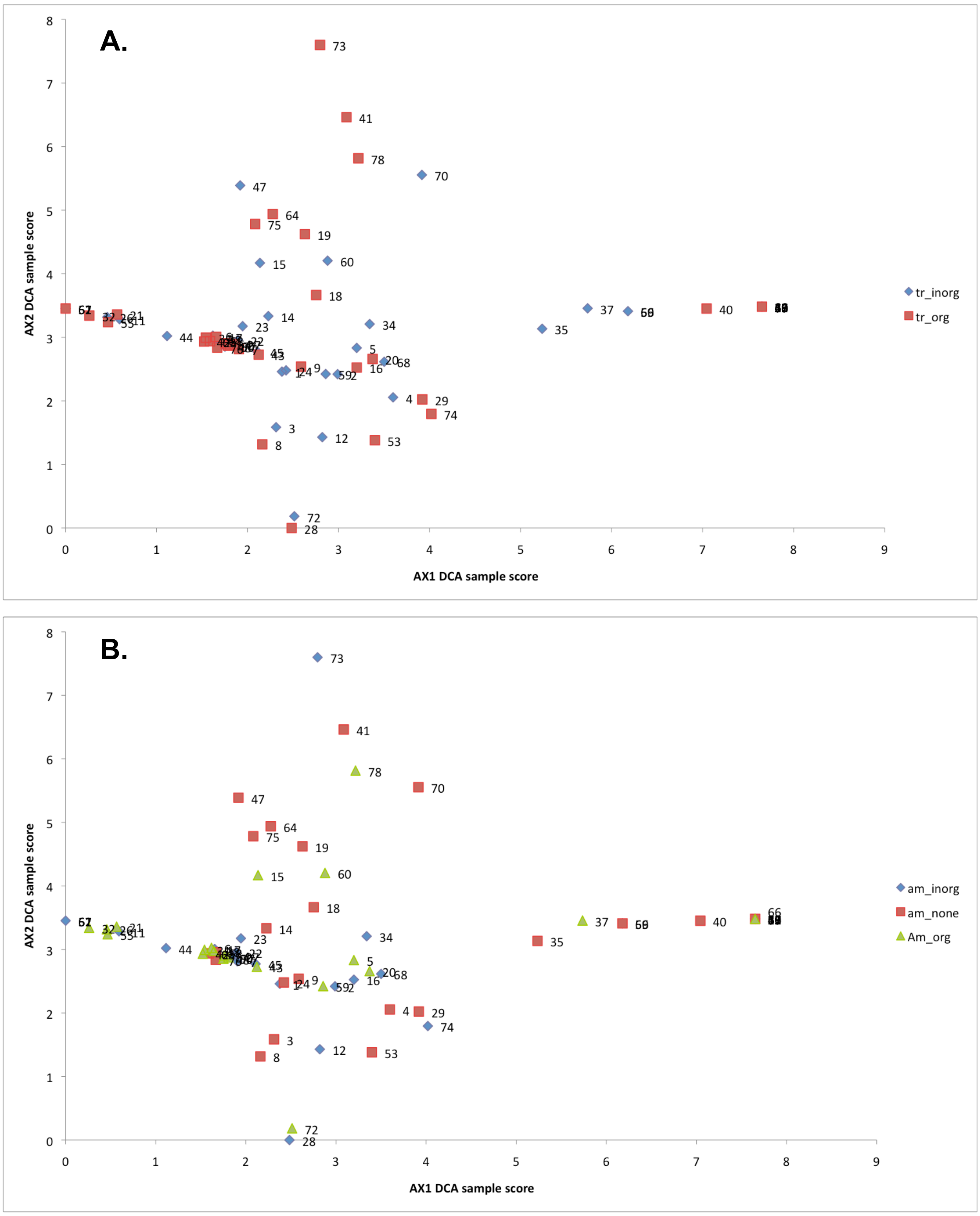

Figure 2.6: Bacterial community first axis sample ordination scores derived from DCA, plotted against second axis ordination scores. A) Samples grouped by original treatment (tr_inorg, mineral fertilizer amendment= Plot 13; tr_org, FYM amendment= Plot 2). B) Samples grouped by laboratory amendment (am_inorg=addition of mineral fertiliser, am_org= addition of FYM, Am_none= no further amendment)

2. 5. Discussion

The aim of this study was to examine the effects on the soil microbial community of amendments of soil samples with inorganic (mineral) or organic (farmyard manure) fertilisers. The soil samples were taken from two plots of the

Palace Leas long-term field trial; namely Plot 2 which receives organic fertiliser and Plot 13, which is amended with inorganic fertilisers. The soil samples were then further treated with either organic or inorganic fertiliser, or left untreated, according to a factorial design. The microbial communities of these samples were examined over 7 weeks, a period equivalent to the life span of the model plant, *Arabidopsis thaliana.* Terminal-Restriction Fragment Length Polymorphisms (T-RFLP) was used to establish the structure of the community in each soil type. Multivariate statistics were then used to identify how the community had changed over time and which of the fertiliser treatments had the greatest effects.

The results showed that changes in bacterial communities were not driven by the original amendment regime on the field, while the laboratory addition of extra fertiliser did have a significant effect. Time also had no effect on the community structure. The fungal community showed no statistically significant response to original field treatment, laboratory amendment or time.

The addition of organic fertiliser is often able to change the microbial soil communities in a significant manner (Widmer et al., 2006). In the Palace Leas Trial, the plots that receive organic fertilisers cluster together on ordination plots, as do the plots that receive conventional fertilisers (O'Donnell et al., 2001). There is a distinct separation between two Plots 2 and 13, when the community level physiological profile was examined. Interestingly, while these plots are separated on an ordination plot, they are closer to each other than to other plots from the field trial. This indicates that there are similarities between the community structure of Plots 2 and 13.

The addition of amendments had a statistically significant effect on the bacterial community structure. The addition of fertiliser amendments have previously been shown to significantly impact the microbial community both in the short-term and in the long-term. Bacterial communities are altered over the short-term by the addition of organic waste products (Bastida et al., 2008). Organic manures have been shown to significantly alter the make up of the community structure of fungi in different soils (Edel-Hermann et al., 2004,Perez-Piqueres et al., 2006). However, it is likely that this is a dose-dependent effect; the addition of lower amounts of compost do not significantly alter the community structure (Crecchio et al., 2001). The effects of amendments on community structure can also be transient (Saison et al., 2006). It is possible that the change seen in the current study was a transient one, although the time period was not long enough to establish this.

Long-term treatment with fertilisers often cause significant divergence in the microbial community (Jangid et al., 2008). Differences in the microbial communities in soil can be directly caused by the management practice (Widmer et al., 2006). This is probably caused by the regular addition of organic fertiliser (Kanchikerimath and Singh, 2001). However, the results of the present study suggest that there is very little difference between the microbial communities in each of the soil types.

It is possible that pH will be having an effect on the community structure. The alteration of pH by the addition of lime (increasing pH) or nitrogen fertiliser (decreasing pH) is capable of significantly altering the microbial community (Kennedy et al., 2004). For example, Kennedy et al found that the addition of lime to increase pH encouraged increased microbial activity, although it may have actually reduced the levels of diversity; addition of nitrogen fertiliser, on the other hand, reduced microbial diversity. The plots at Palace Leas do have different pH values, with Plot 2 being more alkaline than Plot 13, an effect of the addition of superphosphate. As both the fungal and bacterial community show clustering, according to the results of the DCA statistical test, it is possible that pH could be having an effect on the community structure.

Overall, it can be concluded that the major driver of bacterial community in soil from Plot 2 and Plot 13 of the Palace Leas experimental field trial is the addition of further amendment with mineral fertilisers in the laboratory, rather than the different fertiliser regimes carried out in the field.

2. 6. References

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Chapter 3. Effects of Soil Management Practice on Plant Growth

3. 1. Abstract

Plant growth is reliant on the ability to obtain nutrients from the soil. If a soil is unable to provide these nutrients the plant will become stressed, often leading to a reduction in rate of growth before other physical signs become apparent. It is also possible that levels of minerals, such as boron, can lead to the plant becoming increasingly susceptible to disease and insect herbivory. Growth measurements were taken throughout the life cycle of *Arabidopsis thaliana* grown in soil from two plots of the Palace Leas long term field trial. The two plots used were: Plot 2, which is amended with farmyard manure (FYM), and Plot 13, which is amended with inorganic fertilisers. The results indicated that plants grown in the conventionally amended soil grew slower and were smaller than the plants grown in the soil amended with FYM. After 28 days of growth, rosette area was 552.1cm^2 and 418.4cm^2 for plants grown on FYM amended soil and conventionally amended soil, ($p=0.01$), whilst the dry weights were 92.6mg and 72.8mg, respectively ($p=0.044$). Developmental rate was also affected, with a 2-day delay in the onset of flowering in plants grown on the soil amended with mineral fertilisers. Ten days post flowering inflorescence height and leaf number remained significant ($p=0.003$, $F=9.80$, $p=0.017$, $F=6.10$ and $p=0.0015$, for FYM amended and conventionally amended, respectively). At the end point of the experiment (day 48), only leaf area remained significantly different between the two soil types ($p=0.002$, $F=10.16$). Despite rosette dry weight being significantly greater in plants grown on FYM amended soil compared to conventionally grown plants ($p=0.044$, $F=4.24$), neither inflorescence dry weight ($p=0.624$, $F=0.24$) nor seed weight were significantly different. Mineral levels of boron (FYM=0.73ppm, conv=0.31ppm at 30 days), calcium (FYM=177.7ppm, conv=124.9ppm, at 30days), potassium (FYM=331.0ppm, conv=161.5ppm at 30days) and magnesium (FYM=29.5ppm, conv=18.3ppm at 30days) were all significantly higher in plants grown on the FYM amended soil compared to those grown in conventionally fertilised soil. Mineral ratios, such as K/Ca and K/Mg are considered to act as an indicator of plant stress. In this study, both ratios were higher in FYM grown plants,

indicating that the conventional plants were under increased plant stress. Nitrate levels of plants grown on FYM amended soil were significantly greater than for conventionally grown plants. These were 104.593 mM/g ±6.062 and 21.206 mm/g ±9.253 ($p=0.023$) at day 28 for FYM amended soil and conventionally amended soil respectively. At the final time point (35 days) the differences were even greater being 111.77 mM/g ±9.816 and 20.938 mM/g ± 3.418 ($p=0.008$). At no stage was the chlorophyll content significantly different between treatments.

3. 2. Introduction

Since Malthus published his views on population there has been a debate on whether the current food supply can sustain the human population (Malthus, 1826). The Green Revolution of the 1950s led to a large increase in the amount of available food, through new methods of farming such as synthetic fertilisers and pesticides, high-yielding crop varieties and mechanisation of the farm environment. More recently the commercial cultivation of transgenic crops from the mid-1990s (often referred to as the Gene Revolution) has transformed the agricultural landscape once more. Currently 148 million hectares of agricultural land in 29 countries has GM crops grown on it, with significant year-on-year increases (James, 2010). In 2010 an estimated 15.4 million farmers grew transgenic/biotech crops – notably, over 90% or 14.4 million were small resource-poor farmers in developing countries (James, 2010). This increased use of transgenic crops is considered likely to help stabilise food supply, especially in less-developed countries, which are the emerging markets for GM crops. However, it is to be noted that currently 70% of the transgenic crops grown are herbicide tolerant. While this technology provides effective control of weeds from fields, it is less effective for developing countries, due to the additional costs of herbicides (Pimentel and Paoletti, 2009).

It is considered that recombinant DNA techniques are essential to provide enough food for the rapidly increasing human population (Trewavas, 2002, Trewavas, 2004). It is estimated that chemical protection of crops prevents losses by between 20-57%, whilst use of non-chemical means, such as breeding for resistance and adoption of biological pest control are believed to reduce losses by between 10-30% (Pimentel and Paoletti, 2009). However, it is considered that the use of chemical methods have caused significant environmental damage, such as loss of biodiversity, increased resistance in pests to biocides and soil erosion (TIlman, 1999, Matson et al., 1997, Pimentel et al., 1995). Recently there has been a call to move towards more sustainable agricultural systems (Tilman et al., 2002). Systems such as Integrated Farm Management (IFM) use green-revolution techniques while implementing other measures to increase biodiversity, for example, wider field margins (Drummond, 2000, Trewavas, 2004). One of the agricultural practices which claims to be sustainable is that of organic/low input agriculture.

Organic agricultural methods are often considered to be better both for the environment and for human health. In the UK, every major supermarket chain has a line of products produced using organically grown foodstuffs and certified by the Soil Association, the UK's governing body for organic farming. According to the Soil Association, in 2005-6 the market for organic produce increased by 22% and the market was worth £2 billion in 2007. The UK is currently the third biggest organic food market in Europe, after Italy and Germany. However, in developed countries, organically grown crops often show lower yields than crops grown using conventional methods. In the UK, in a Co-Operative Society funded study, yields of organic oats and wheat were 81% and 68% of their conventional counterparts (Leake, 1999). Long term field trials also show a decrease in the yields produced when using organic and biodynamic techniques. These techniques saw an average 20% drop in yield. However, this was coupled with a 30-50% decrease in the input of nutrients (e.g. N, P, K) indicating that organic agriculture is more effective in resource utilisation (Mäder et al., 2002). A recently published review of yields for organic and conventional agricultural practices indicated that there was a decrease in yields from organic crops. Organic grain yields were an average of 93% of conventional, while organic leguminous crops were 89% of conventional (Badgley et al., 2007). These examples indicate that organic yields cannot yet reach the levels produced by conventional practices. It is possible that this disparity will become greater with the wider use of transgenic crops.

Comparative yield studies indicate that a larger amount of land would be needed to produce the same amount of food using organic farming techniques

(Trewavas, 2002). While nutrient input can be lower on organic farms, other energy inputs are likely to be significantly higher. Using figures for fossil fuel use derived from studies by Leake (Leake, 2000, Leake, 1999) the energy costs per tonne of yield were calculated. Organic farming systems used 200kWh per tonne of yield, while conventional farming used 140kWh per tonne of yield, a significant decrease, especially at current fuel prices (Trewavas, 2004). Most of this additional energy is needed for increased ploughing and mechanical weed removal. Furthermore, both of these practices can be damaging to the environment, as they disrupt soil invertebrates such as worms (Trewavas, 2001).

The use of organic farming techniques is thought to provide some measure of resistance against both disease and insect predation. The application of composts has been linked to the prevention of soil-borne pathogens at both the glasshouse and field level (Drinkwater et al., 1995, Zhang et al., 1998). There is also evidence that these applications can help prevent against foliar diseases through the mechanism of Systemic Acquired Resistance (SAR). In SAR, the plant's defences are conditioned to further attack by pathogens, due to a prior infection (Vallad and Goodman, 2004). It is likely that the presence of possible pathogenic bacteria in compost amendments is activating the pathways in plants involved in the induction of SAR. However, it is also likely that the deliberate induction of SAR will result in increased physiological costs to the plant, as it diverts resources to the activation of this system (Vallad and Goodman, 2004).

While SAR protects mainly against pathogens, there is some evidence that there is cross-talk between the disease resistance pathways and the insect resistance pathways, mainly in the forms of jasmonic acid and salicylic acid signalling (van Poecke and Dicke, 2004). This cross talk can therefore help to provide some resistance against insect predators from the application of composts. However, there is also some evidence to suggest that pathogen and insect resistance can be provided through non-genetic means, namely the maintenance of a balance of micronutrients. The idea outlined above was first presented in 1985 by Francis Chaboussou. He believed that the relationship between plant and parasite was primarily based on the nutritive status of the plant. A deficiency in a nutrient, for example boron, would cause a decrease in the amount of protein synthesis, which in turn would lead to a build up of amino acids, which allows for increased nutrition for parasites of the plant (Chaboussou, 1985). Chaboussou believed that the deficiencies were the direct result of chemical fertiliser and pesticide use. At the time of its publication this theory, called trophobiosis, was ignored.

However, there have been subsequent studies, which appear to show a decrease in insect predation in organically grown plants, linked to lower levels in micronutrients. For example, there were lower levels of European Corn Borer (*Ostrinia nubilalis;* ECB) oviposition on maize (*Zea mays*) plants grown on soil collected from organic farms than soil from conventional farms. This effect was attributed to the ratios and levels of minerals (Phelan et al., 1995, Phelan et al., 1996). There was also evidence at a field level of decreased abundance and fecundity in the Colorado potato beetle (*Leptinotarsa decemlineata* (Say)) on organically grown potatoes (Alyokhin et al., 2005, Alyokhin and Atlihan, 2005). However, there is also evidence that organic amendment of soils does not affect Colorado potato beetle negatively and increased organic amendment shortens the larval development times (Boiteau et al., 2008)

Previous studies using soil from Palace Leas, a long-term field trial at Cockle Park Experimental Farm, Newcastle University, demonstrated that the fully organic plot (Plot 2) has consistently cropped higher levels of hay than the fully conventional plot (Plot 13). The mean yield for the period 1897-1984 for Plot 2 is 5920±1714kg ha^{-1}, while for Plot 13 it is 4415±1271kg ha^{-1} (Coleman et al., 1987).

The present study, with *Arabidopsis thaliana* as a model plant, was designed to establish whether the effects seen on hay yield from the Palace Leas Plots was also observed under controlled environmental conditions. In addition to an analysis of soil type on plant growth and yield, the present study was also designed to investigate the effects of the management practices on total chlorophyll levels, nitrate levels and inorganic mineral levels within the plant.

The experiment was designed to identify the effects of two different management practices (organic amendment using farmyard manure and inorganic amendment using mineral fertilisers) on the mineral levels and subsequent growth rate of *Arabidopsis thaliana*. Soil samples from the Palace Leas Experimental hay plots were used in this experiment. Since this site has only been treated with fertiliser for over 100 years, it provides ideal controlled conditions for determining the effects of amendment on plant growth. A map marking the positions of these two plots can be seen in Figure 3.1, while Table 3.1 provides details of the fertiliser input of the two plots (Plot 2 and Plot 13).

Arabidopsis thaliana was selected as the model plant species as it's genome has been fully sequenced and annotated. The plant is small and fast growing and growth is easily measured, which makes it ideal for experiments of this type.

3. 3. Materials and methods

3. 3. (a) Biological materials

3. 3. (a) (i) Soil collection and processing

Soil samples were collected from the Palace Leas long-term field trial at Cockle Park Farm, Newcastle University (established in 1896). Samples were taken from two plots in this trial, Plot 2, which is amended with farmyard manure (FYM) and Plot 13, which is amended with inorganic fertilisers (Table 3.1, Figure 3.1, Coleman et al., 1987). Samples were taken using an auger to a depth of 20cm and from across the length of the plot. Samples were taken within 2 weeks of treatment application. The soil cores were sieved through a 2mm sieve to remove plant debris and to achieve homogenisation. Storage was at 4ºC until required.

Table 3.1. Amendment treatments for Palace Leas Meadow Hay Plots, Cockle Park Farm, Northumberland. (This site has only been treated with fertilisers for over 100 years)

Plot No.	**Rotation year**	**FYM (t ha^{-1})**	**Amendment (Kg ha^{-1})**		
			N	**P_2O_5**	**K_2O**
1		20	17	30	34
2		20			
3	1	20			
	2		17	30	34
4	1	20			
	2				
5	1	40			
	2		17	30	34
	3		17	30	34
	4		17	30	34
6					
7			35		
8				60	
9					67
10			35	60	
11			35		67
12				60	67
13			35	60	67
14			100	66	100

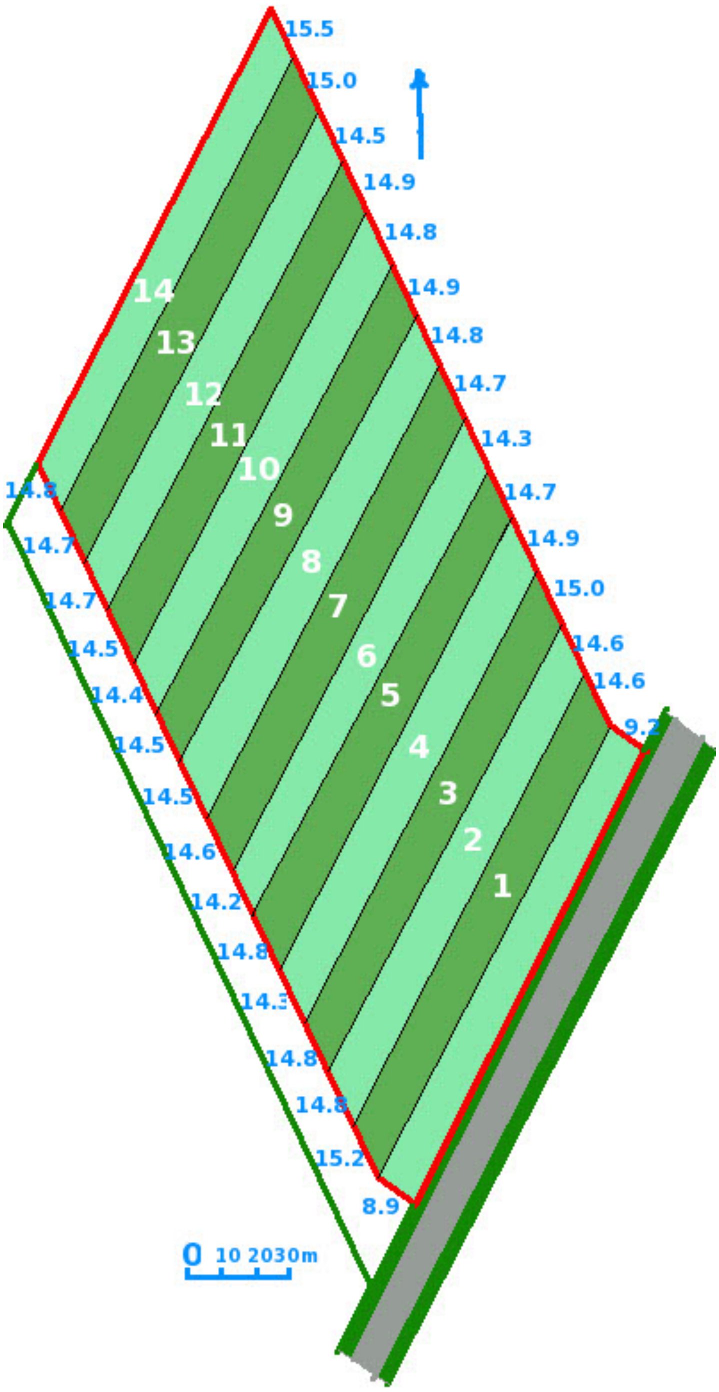


Figure 3.1. Plan of the Palace Leas Meadow Hay Plots, Cockle Park Farm, Northumberland (Grid Reference; NZ 201 915) (Personal communication from R. Shiel)

3. 3. (a) (ii) Plant growth conditions

Arabidopsis thaliana (ecotype C24) seeds were placed on moist filter paper and vernalised at 4ºC for 48 hours. Soil was prepared for potting by mixing with perlite to a final concentration of 30% so as to allow adequate drainage. 7.5cm pots were filled and between 2 and 5 seeds were placed onto the surface of the soil. To allow germination, humidity was increased using catering film taped to the pots.

All plants were grown in a controlled environment room with a 16/8hr day/night light cycle and a 22/20ºC day/night temperature cycle. Illumination averaged at 150µmol s^{-1} m^{-2}. Sufficient water was applied throughout the course of the growing period to keep the soil moist. Thirty replicates per treatment were used to allow accurate statistical analysis (see section III. 2. (b) (v)).

3. 3. (b) Methods

3. 3. (b) (i) Plant growth measurements

Measurements of growth were taken once the plants had reached growth stage 1.02 (2 rosette leaves greater than 1mm in length, from Boyes et al., (2001)). This was reached 8 days after germination and this point was established as day zero. The measurements taken were leaf number, rosette diameter, rosette area, rosette perimeter, inflorescence height, silique number and mean silique length (taken from an average of 5 siliques) (Boyes et al, 2001).

To establish accurate values for these measurements, digital images of each plant were taken every 2 days from day zero. These images were then processed using ImageJ (National Institutes of Health, Washington DC, USA). For each image the workflow consisted of converting from colour to black and white, then processing to form a binary image. Calibration was achieved using a scale provided in each image and the calibration function in the programme. All measurements were then taken using the appropriate function in imageJ.

At the end of the growth period, all rosettes were separated from the inflorescences and dried in a desiccator before weighing. Seeds were sieved from siliques and weighed separately after the inflorescences were weighed.

3. 3. (b) (ii) Determination of leaf chlorophyll levels

Samples were taken weekly from day zero to determine chlorophyll levels. Leaf tissue was taken from plants established at the same time as the previously described experiment. The extraction method was adapted from Lichtenthaler (1987). Briefly, 0.30g of leaf tissue was ground in 4ml of 80% acetone and centrifuged at 13,000g for 5 minutes at room temperature. Absorbances of the supernatant were then read at 647nm, 663nm and 750nm using a quartz microtitre plate in a Spectromax spectrophotometer. The extraction was carried out under natural light, out of direct sunlight, to limit degradation of pigments.

The value obtained from the 750nm reading was subtracted from the other absorbance values and these net values were then substituted into

Equation 1: chla

$\text{chla} = 12.25A_{663} - 2.79A_{647}$

Equation 2: chlb

$\text{chlb} = 21.5A_{647} - 5.10A_{663}$

Equation 3: chl(a+b)

$\text{chl}(a+b) = 7.15A_{663} + 18.71A_{647}$

chla = leaf chlorophyll a content (µg ml-1), chlb = leaf chlorophyll b (µg ml-1), chl(a+b) = total leaf chlorophyll content (µg ml-1), Ax = absorbance at x nm - absorbance at 750nm.

3. 3. (b) (iii) Tissue nitrate levels

Tissue nitrate levels were estimated using an adapted spectrophotomeric method. Briefly, 0.1g of finely ground plant tissue was brought to the boil in 1ml of water to extract all the available tissue nitrate into the water. This sample was then made up to 5ml with ultrapure water. 1.25g of Nitratest powder (Palintest, Gateshead, UK) was added, the sample was shaken and 0.5g of a Nitratest tablet was added. After letting the sample develop for 5 minutes, a further 0.5g of Nitricol powder was added and the sample was left to develop colour for 15 minutes. Samples were then read in a Spectromax Spectrophotometer at 540nm.

To establish the levels of nitrate present, the absorbance values of each sample were compared to a standard curve prepared from dilutions of a 1M sodium nitrate solution (Standard curve r^2=0.998). Samples were taken at 7, 28 and 35 days. Three biological replicates were taken per treatment per time point and three technical replicates were taken from each biological replicate to give a total of nine replicates per treatment per time point.

3. 3. (b) (iv) Inorganic mineral levels

Inorganic mineral levels were measured using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) to identify levels of three inorganic minerals: boron, zinc and magnesium. Briefly, finely ground plant material was digested using concentrated (15.8M) nitric acid overnight; the acid was added at a ratio of 1ml acid per 1g tissue. The resultant paste was then diluted to 4 times the original volume with pure water. Each sample was then submitted to Newcastle University's Advanced Chemical and Materials Analysis service. Results were returned in ppm. Three time points were used at 7 days, 20 days and 30 days.

3. 3. (b) (v) Statistical analysis

All data were assessed using the Anderson-Darling test to establish whether these data were parametric. Parametric data were analysed using one way ANOVA with confidence limits of 95%. Non Parametric data were analysed using Mann-Whitney U test, with confidence limits of of 95%. All chlorophyll, mineral and nitrate data were assessed using Student's t-test, again with 95% confidence limits.

3. 4. Results

3. 4. (a) Effect of soil type on plant growth parameters

Arabidopsis thaliana plants were grown on soil from either a FYM amended experimental plot or a conventionally amended plot for a period of 55 days, from seed to senescence. The results obtained show that plants grown on soil from Plot 2, the FYM amended plot from the Palace Leas field trial, were repeatedly significantly larger than plants grown on soil from the conventionally amended Plot 13.

After 8 days, the diameters were very significantly greater in the plants grown on soil from Plot 2 (p=0.000, F=46.19). Similarly this was true for both area (p=0.000, F=38.47) and rosette perimeter (p=0.000, F=79.43). Leaf number, which was analysed using the Mann-Witney U test, was also significantly greater in plants grown under the FYM fertilised regime (p=0.0021).

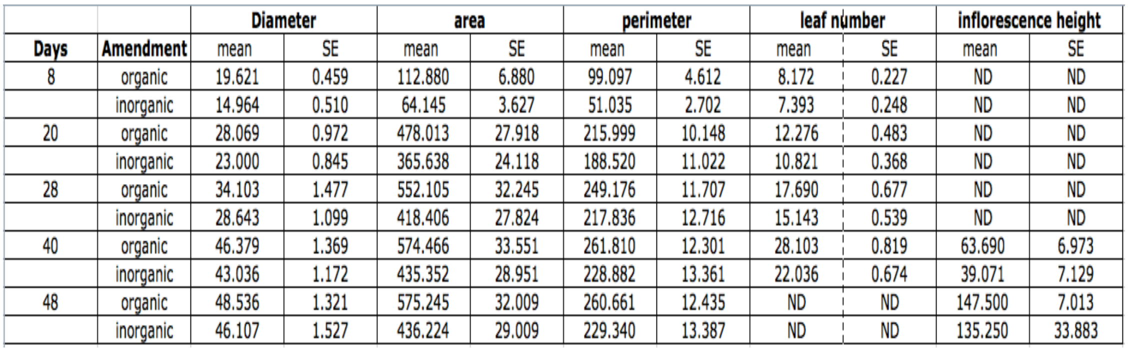
A similar growth pattern is seen after 20 days. For most parameters measured, this was the stage at which growth rates were seen to decline (Figures, 3.2 3.3, 3.4, 3.5). Again, FYM grown plants showed significant differences from the conventionally grown plants with significant values for rosette diameter, rosette area and leaf number being p=0.000 (F=15.39), p=0.0027 (F=9.22) and p=0.0021 respectively. However, by this point, perimeter was no longer significant (p=0.072, F=3.37).

Soil management not only affected plant size, but also developmental rate. Flowering started after 30 days for the plants grown on the FYM amended soil and was 2 days later for plants on the conventional soil. After 40 days area, inflorescence height and leaf number remained significant (p=0.003, F=9.80, p=0.017, F=6.10 and p=0.0015, respectively).

At the end point of the experiment (day 48), only leaf area remained significantly different between the two soil types (p=0.002, F=10.16). Diameter (p=0.238, F=1.42), inflorescence height (p=0.725, F=0.13) and perimeter (p=0.095, F=2.89) were all non-significant by this stage (values in Table 3.3)

Since the effect of soil type on yield is of major importance, the average dry weights of the inflorescence, seed weight and rosette were recorded (Figure 3.5). The results demonstrated that whilst rosette dry weight was significantly greater in FYM grown plants than conventionally grown plants (p=0.044, F=4.24), inflorescence dry weight was not (p=0.624, F=0.24). Seed weight was also not significant.

Table 3.2: Values for plant measurements over time. Plants were either grown on soil amended with organic FYM or inorganic mineral fertiliser; there were 30 replicates/treatment. All units mm except for leaf number (no units) and area (mm^2).



		Diameter		area		perimeter		leaf number		inflorescence height	
Days	**Amendment**	mean	SE	mean	SE	mean	SE	mean	SE	mean	SE
8	organic	19.621	0.459	112.880	6.880	99.097	4.612	8.172	0.227	ND	ND
	inorganic	14.964	0.510	64.145	3.627	51.035	2.702	7.393	0.248	ND	ND
20	organic	28.069	0.972	478.013	27.918	215.999	10.148	12.276	0.483	ND	ND
	inorganic	23.000	0.845	365.638	24.118	188.520	11.022	10.821	0.368	ND	ND
28	organic	34.103	1.477	552.105	32.245	249.176	11.707	17.690	0.677	ND	ND
	inorganic	28.643	1.099	418.406	27.824	217.836	12.716	15.143	0.539	ND	ND
40	organic	46.379	1.369	574.466	33.551	261.810	12.301	28.103	0.819	63.690	6.973
	inorganic	43.036	1.172	435.352	28.951	228.882	13.361	22.036	0.674	39.071	7.129
48	organic	48.536	1.321	575.245	32.009	260.661	12.435	ND	ND	147.500	7.013
	inorganic	46.107	1.527	436.224	29.009	229.340	13.387	ND	ND	135.250	33.883

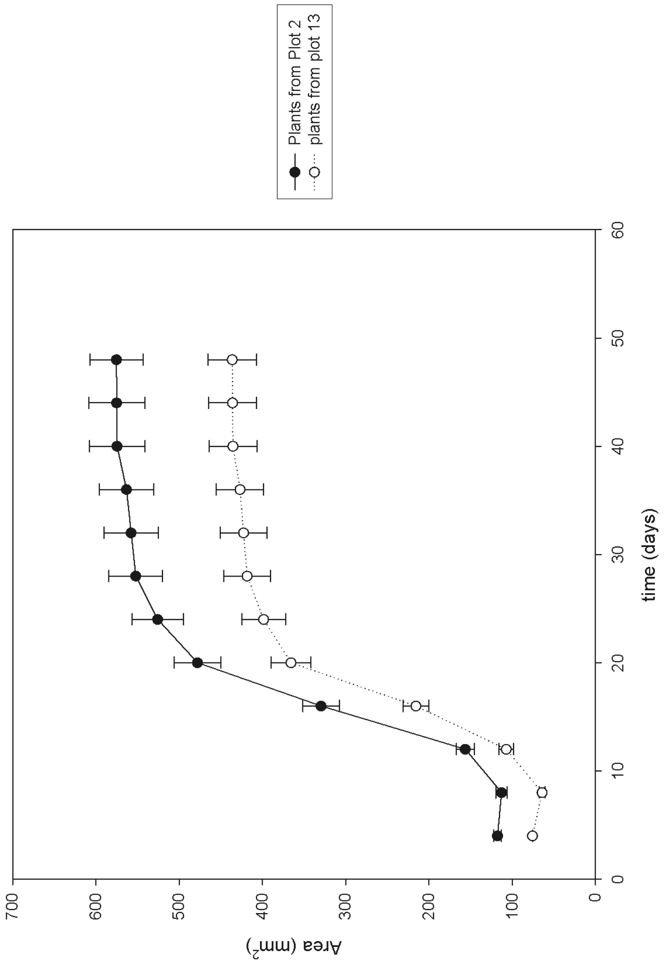


Figure 3.2: Mean rosette areas of *Arabidopsis thaliana* over time. Soil from Plot 2 was amended with FYM, while soil from Plot 13 was amended conventionally. n=30

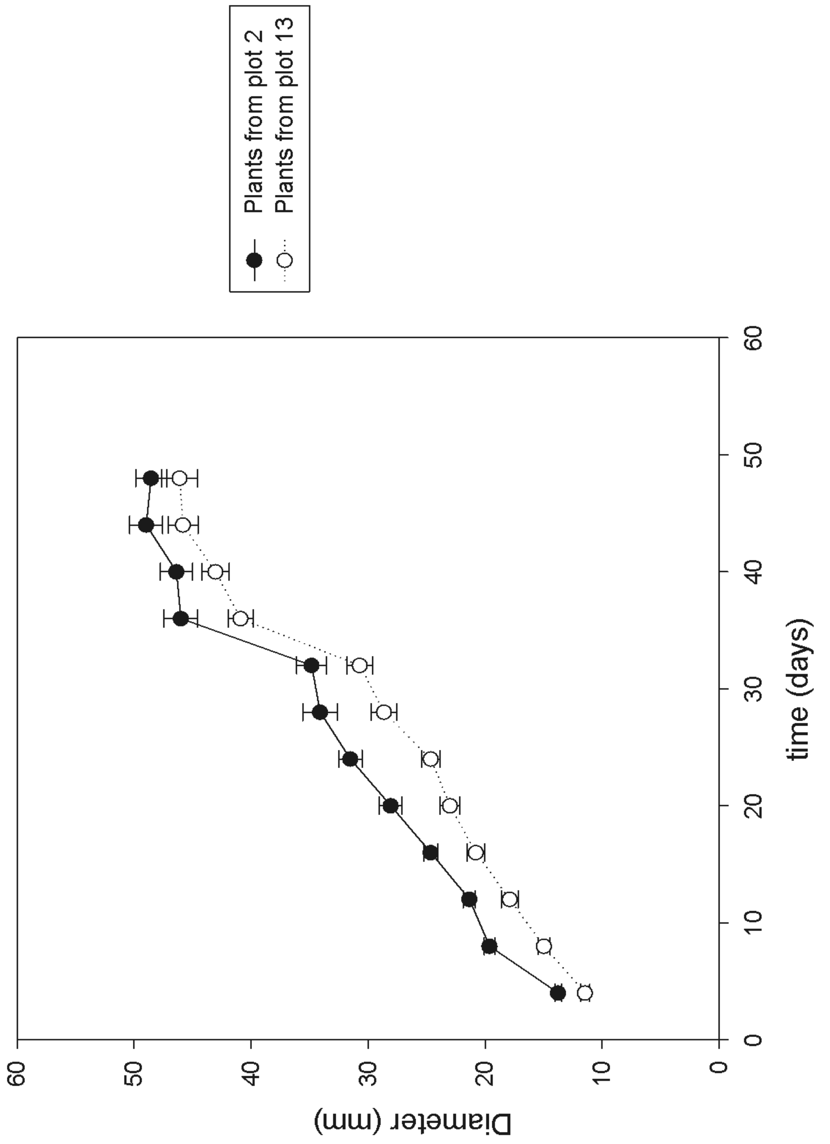


Figure 3.3: Changes in mean rosette diameter of *Arabidopsis thaliana* over time. Soil from Plot 2 was amended with FYM, while soil from Plot 13 was amended conventionally. n=30

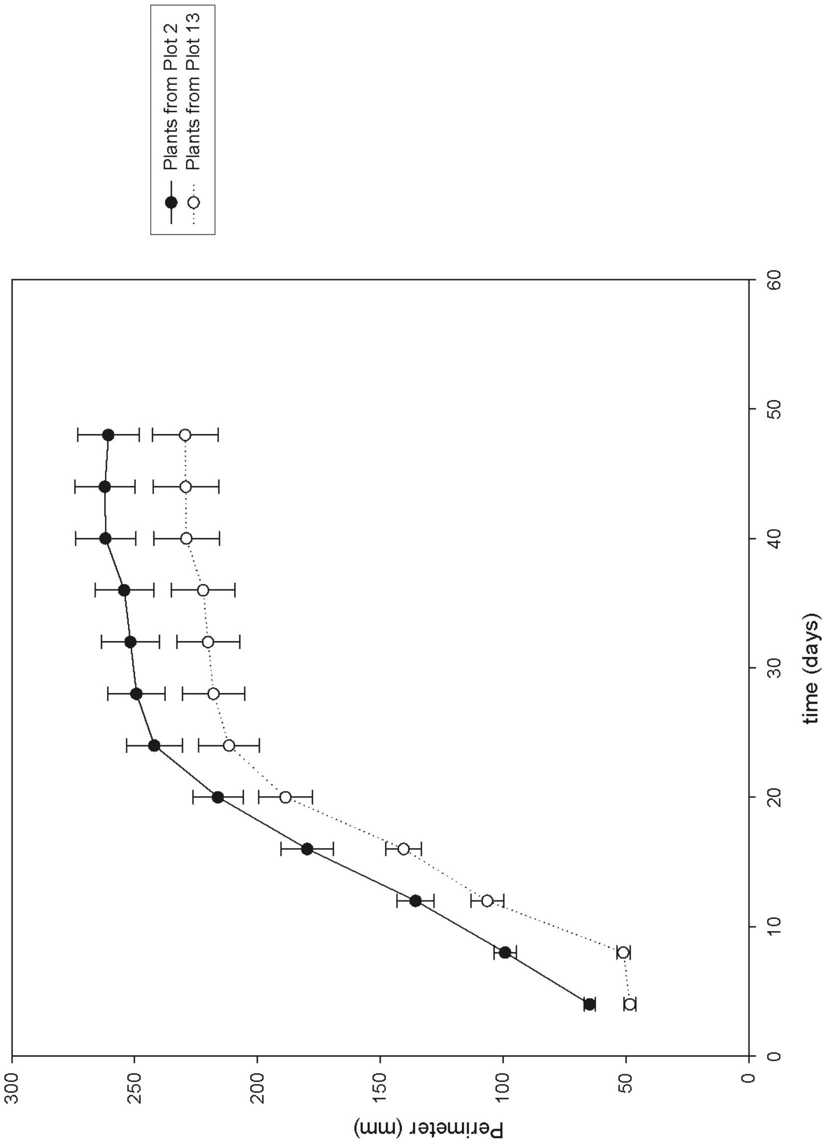


Figure 3.4: Changes in rosette perimeter of *Arabidopsis thaliana* over time. Soil from Plot 2 was amended with FYM, while soil from Plot 13 was amended conventionally. n=30.

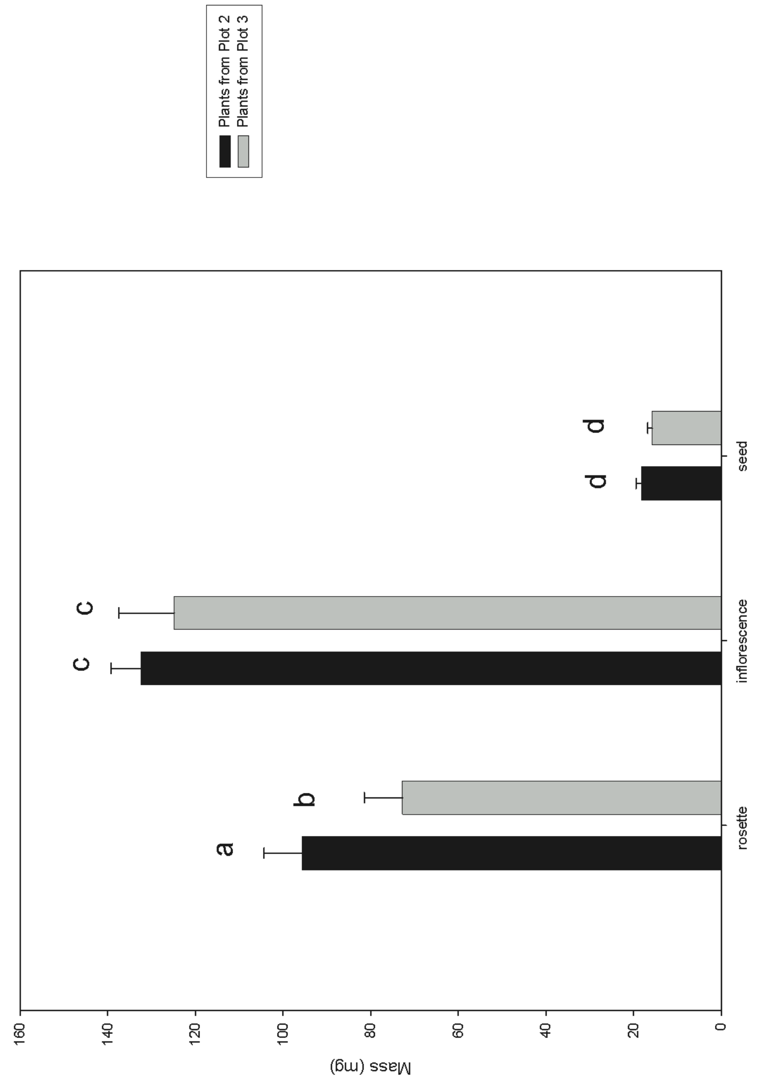


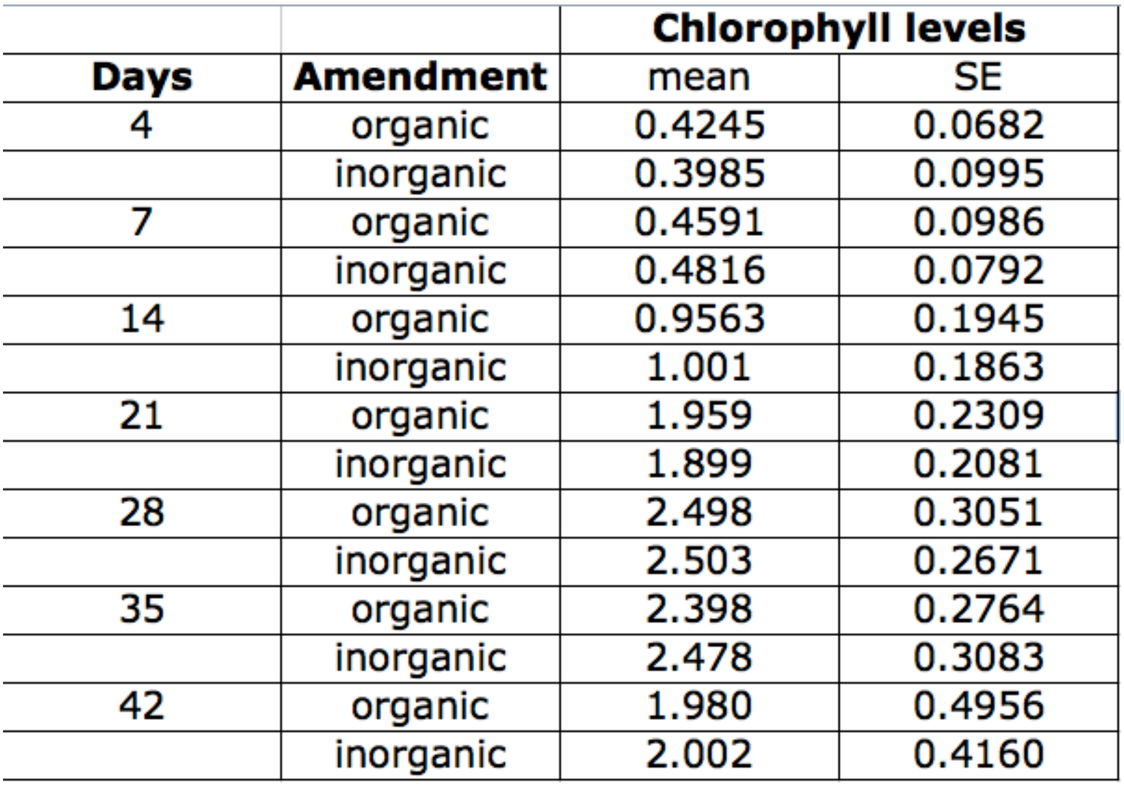
Figure 3.5: Dry Weights of rosettes, inflorescences and seed yield from *Arabidopsis thaliana* grown on soil from either Plot 2 (amended with FYM) or Plot 13 (amended with mineral fertilisers). n=30. Significant differences are indicated with letters, where the same letter indicates no difference.

3. 4. (b) Effect of soil type on chlorophyll levels

Total chlorophyll levels were measured 4 days after germination and then at weekly intervals throughout the trial with the maximum levels being reached after 28 days. Conventionally grown plants had an average of 2.503 (±0.267)mg/g, while plant grown on FYM amended soil had an average of 2.498 (±0.305)mg/g. By the last time point taken, after 42 days, chlorophyll levels had decreased to 2.00 (±0.416) mg/g and 1.980 (±0.496) mg/g in plants grown on conventionally amended soil and FYM amended soil, respectively (Table 3.3, Figure 3.6).

As can be seen in Table 3.3 and Figure 3.6, the differences between the two amendment techniques were insignificant.

Table 3.3: Chlorophyll levels in *Arabidopsis thaliana* plants grown in either conventionally amended soil (Plot 13) and or FYM amended soil (Plot 2) over time. There were 3 replicates/treatment. All values in mg/g



		Chlorophyll levels	
Days	**Amendment**	mean	SE
4	organic	0.4245	0.0682
	inorganic	0.3985	0.0995
7	organic	0.4591	0.0986
	inorganic	0.4816	0.0792
14	organic	0.9563	0.1945
	inorganic	1.001	0.1863
21	organic	1.959	0.2309
	inorganic	1.899	0.2081
28	organic	2.498	0.3051
	inorganic	2.503	0.2671
35	organic	2.398	0.2764
	inorganic	2.478	0.3083
42	organic	1.980	0.4956
	inorganic	2.002	0.4160

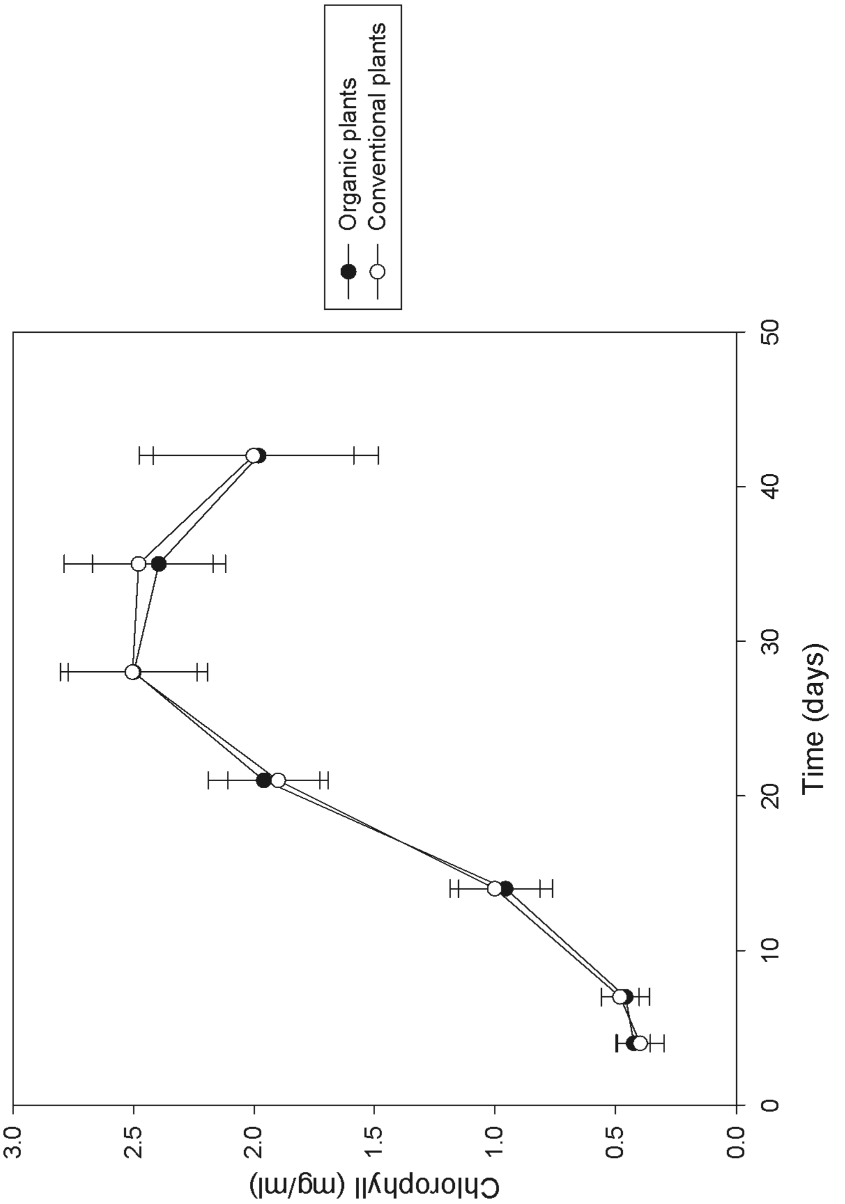


Figure 3.6: Chlorophyll levels over time in *Arabidopsis thaliana* plants grown in conventional (Plot 13) and FYM (Plot 2) treated soil. Chlorophyll values are in mg/g, n=3.

3. 4. (c) Effect of fertiliser regime on tissue nitrate levels in *Arabidopsis thaliana*

Nitrate levels are often discussed as being an indicator of food quality. In this study these levels were measured at 3 time points throughout the course of the growing period (Table 3.4). At the first time point, after 7 days, nitrate levels were not statistically different between the two treatments,, with plants grown on FYM amended soils having an average of 42.428mM per gramme wet weight (±9.833), while plants grown on conventionally fertilized soil having an average of 31.334mM/g (±9.051). At the second time point, 28 days, the plants grown in the FYM amended soil (104.593mM/g ±6.062) had a statistically higher level of nitrate than the conventionally grown plants (21.206mM/g ±9.253; p=0.023). By the third time point, the levels of nitrate in plants on the FYM amended treatment (111.774mM/g ± 9.816) showed an even larger difference compared to the conventionally grown plants (20.938mM/g ± 3.418; p=0.008)

Table 3.4: Nitrate levels in *Arabidopsis thaliana* plants grown in FYM (Plot 2) and conventionally (Plot 13) amended soils. Values are in mM per gramme wet weight. * indicates $p < 0.05$, ** indicates $p < 0.01$. n = 3

		Nitrate Level		
Day	**Amendment**	mean	SE	
7	organic	42.428	9.833	
	inorganic	31.334	9.051	
28	organic	104.593	6.062	*
	inorganic	21.206	9.253	
35	organic	111.774	9.816	**
	inorganic	20.938	3.418	

3. 4. (d) Effect of fertiliser regime on levels of inorganic minerals in *Arabidopsis thaliana* plants

The levels of four different inorganic minerals were analysed to identify how they were affected by the two different methods of amendment used. The minerals examined were potassium, calcium, magnesium and boron. After the samples were dried, there was inadequate tissue to do more than one biological replicate per time point, so the samples were pooled. Three technical replicates per time point were performed.

Overall, levels of each mineral were significantly greater in plants grown in FYM amended soil (Plot 2) compared to those grown on soils amended with conventional fertilisers (Plot 13). Both Mg and B had consistently significantly higher levels in the plants from FYM amended soils over all three time points (Table 3.5). For K and Ca the first and third time point both showed significantly higher levels in plants grown in soil from Plot 2, while the differences at the second time point are not significant (Table 3.5). K/Ca ratios are significantly higher in plants from the FYM amended soils after 7 days. However, this was reversed after 20 and 30 days, with plants grown on soil from Plot 13 having a higher ratio than the plants grown on Plot 2 (Table 3.5). The K/Mg ratio is significantly increased in plants grown on FYM amended soil after 20 and 30 days.

Table 3.5: Values of mineral levels in Arabidopsis thaliana plants grown on conventionally amended soil (Plot 13) and FYM amended soil (Plot 2). All units for mineral values are ppm. * indicates statistical significance $p < 0.05$, ** indicates statistical significance $p < 0.0$; n= 3

		Mineral											
		K			**Mg**			**Ca**			**B**		
Day	**Amendment**	Mean	SE		Mean	SE		Mean	SE		Mean	SE	
7	organic	107.60	1.03	**	20.380	0.05	**	189.770	1.33	**	0.6656	0.0035	**
	inorganic	89.40	0.82		15.800	0.28		107.970	0.8		0.2896	0.001	
20	organic	174.00	1.35		15.030	0.05	**	97.140	0.13		0.4487	0.0027	**
	inorganic	121.90	0.97		16.140	0.33		95.300	0.75		0.2919	0.0049	
30	organic	331.03	0.88	**	29.540	0.29	**	177.700	0.5	**	0.7302	0.1005	*
	inorganic	161.53	1.86		18.350	0.22		124.990	0.84		0.3138	0.0038	

		Ratios					
		K/Ca ratio			**K/Mg ratio**		
Day	**Amendment**	Mean	SE		Mean	SE	
7	organic	0.567	0.01	**	5.28	0.05	**
	inorganic	0.828	0.01		5.66	0.06	
20	organic	1.791	0.01	**	11.58	0.09	**
	inorganic	1.279	0.00		7.557	0.12	
30	organic	1.863	0.01	**	11.21	0.14	**
	inorganic	1.292	0.01		8.804	0.06	

3. 5. Discussion

The present study investigated the effects of farmyard manure (FYM) and conventional amendment regimes on *Arabidopsis thaliana* as a model system. Effects studied included physical growth characteristics, such as leaf area, rosette diameter and plant dry weight to establish how treatment regime affected the size and yield of the plant. Chlorophyll levels were measured to examine the effect of amendment on the plant's level of photosynthetic capacity and as an indicator of the plant's nitrogen status. Nitrate levels were also measured, as these have been implicated as a risk factor in food quality, which are likely to be influenced by soil management practices. The levels of four minerals were also measured. Boron has been identified as a mineral that is often deficient in conventionally grown plants, whilst potassium, calcium and magnesium were measured as the K/Ca and K/Mg ratios have been implicated in providing an identifier of plant health status (Chaboussou, 1985).

The physical data collected during the course of this study indicated that on FYM amended soil, *A. thaliana* grew larger during the initial and middle growth stages, but by the end of the growing period there was little significant difference between the two treatment types. When the plant entered senescence, only total leaf area was still significantly different between the two treatments. Dry weights, which could be considered the "yield" of *Arabidopsis*, only show significance in the rosette weights, with the plants grown on the FYM amended soil being higher. Seed and inflorescence dry weights were not significantly different. Yields of hay at the Palace Leas field trial for the two plots in this experiment show that Plot 2 produces significantly more hay than Plot 13. Plot 2, the FYM amended plot produces an average 6496kg ha^{-1} (±2063), while plot 13, the conventionally amended plot, produces an average of 4415kg ha^{-1} (±1271) (Coleman et al., 1987). While the data presented here is supported by the yield data from Palace Leas, other published studies indicate that organic management cannot produce the yield of crops that can be maintained from conventional management practices. It is believed that one of the causes of this is due to the lack of available nutrients for crops from organic amendment regimes. Organic soils should contain around 300kg ha^{-1} of nitrogen, while analysis has shown that the amount freely available for use by the plant is only 50kg ha^{-1} (Berry et al., 2002). Nitrogen is often available at the wrong time needed for rapid plant growth. Plants go through a rapid increase in growth rate in the spring and so have a significant need for nitrogen at this point. This is allowed for in best practice conventional farming, as highly soluble fertilisers are applied at this point, allowing for rapid uptake by crops (Trewavas, 2004). Studies featuring yield comparisons appear to support this. Leake, (1999) showed significantly reduced yields in grain crops over the term of their study. The Boarded Barns field trial also had significantly reduced yields of winter wheat, the main grain crop in the UK. The yields from organically grown crops averaged 50% less than the conventional yields from fields on the same experimental farm (Aventis Crop Science, 2000). There is, however, evidence that regular applications of organic amendment can increase yields. As mentioned already, the hay yields of these plots from the Palace Leas site show that organic amendment produces the highest yields.

Table 3.6. Extractable phosphorus (mg kg^{-1}), extractable cation content (m mol$_c$ kg^{-1}) and pH in selected plots of the Palace Leas field trial.

Plot	K (april)	K (Oct)	P	Ca	Mg	pH
2	5.1	3.89	126	113.6	14.1	5.4
13	5.69	4.81	51	63.6	13.7	5.0

The two plots at Palace Leas have differing pH levels, with Plot 13 being slightly more acidic than Plot 2, a difference that has been caused by the use of ammonium sulphate as the nitrogen source (Table 3.6, Shiel, Personal Communication). As the pH of soil decreases, mineral availability is altered in a complex pattern. However, due to the small difference (0.4 units) in pH between the two plots, it is unlikely that this has had a major effect on the plant growth. On plots where there is a significant difference in pH levels, (for example Plots 11 and 12) there is also a significant difference in the yield of hay, although other factors (such as the lack of phosphorous amendment on Plot 11, are likely to also have a significant effect on yield (Shiel, personal communication).

The two long term field trials at Rothampsted Research also show that organic amendment is capable of producing yields similar to conventional amendment. On the Broadbalk trial sections where wheat is grown continuously, the FYM treated plots produce equivalent yields to sections treated with a high level of nitrogen amendment. On the Hoosfield trial which features barley as the main crop, the FYM amended plots have consistently out-produced the conventional ones in terms of yield (Rothamsted Research, 2006).

The System of Rice Intensification (SRI) also has shown significant yield increases for subsistance farmers in developing countries, through, in part, the use of composted organic matter (Uphoff, 1999). In these systems, farmers apply large levels of composted matter, up to 40t ha^{-1}, to achieve these higher yields. Plot 2 at Palace Leas receives 20t ha^{-1} of FYM per annum, which is also a high level of amendment, as is the Broadbalk trial, which receives 35t ha^{-1}. These studies suggest that high levels of organic fertiliser are able to compensate for any nitrogen deficiencies encountered by crops grown under organic regimes.

Chlorophyll is often used as an indicator of a crop's nitrogen utilised status, as up to 70% of the plant's nitrogen is stored in the chloroplast, so there is often a correlation between chlorophyll levels and leaf nitrogen (Madakadze et al., 1999, Zhao et al., 2007). In the study reported here, there was no significant difference in the amount of total chlorophyll present in both FYM amended or conventionally amended *A. thaliana.* This indicates that the nitrogen status of plants grown in soil from both treatments were similar.

Boron (B) levels were significantly higher in *A. thaliana* plants grown on FYM amended soils. These plants also had a higher rate of growth than the plants grown on the conventionally amended soil. It is possible that the lower levels of B present in the plants grown on conventionally amended soil contributed towards the decreased growth rate seen here. FYM appears to increase B concentration in sunflower, by increasing availability (*Helianthus annuus*) (Sharma et al., 1999). At the highest level of FYM application, there was a significant increase in the concentration of B in the plant. The addition of FYM may also help moderate the loss of B in acidic soils, as it can increase the adsorption of B to the soil, preventing leaching (Sharma et al., 2006). The Palace Leas plots used in this study are both acidic (Coleman et al., 1987). It is possible that the addition of FYM to Plot 2 is allowing a larger pool of B to be present for uptake by the plants. Ruiz et al (1998) showed significant increases in leaf dry weight, protein content and amino acid content in tobacco (*Nicotiana tabacum* L.) with increases in B amendment.

Nitrate (NO_3^-) levels in this study were, for the second and third time points, significantly higher in *A. thaliana* grown on FYM amended soil. NO_3^- levels are often used as an indicator of food quality, as high levels of NO_3^- within the plant can cause health problems in humans and animals. Inorganic fertilisers such as ammonium and potassium nitrate often cause high levels of NO_3^- within plants, while FYM and non-nitrate containing fertilisers, such as ammonium sulphate are associated with lower levels of NO_3^- (Turan and Sevimli, 2005). In this study, ammonium sulphate was considered a more appropriate fertiliser for giving low NO_3^- levels within cabbage. Plot 13 at Palace Leas receives nitrogen in the form of ammonium sulphate and calcium nitrate, and it is possible this explains the lower levels of NO_3^- present in *A. thaliana* grown on soil from that plot. However, it is not possible to determine whether this is the direct cause from the results presented here. Currently, there is only limited data for nitrogen levels in the Palace Leas field trial, particularly for the two plots used in this experiment. Nitrogen levels have been shown to be higher on the more acidic plots (Shiel, 1986). In the current study, plot 13, which received inorganic fertiliser, is more acidic.

A further possible explanation for the higher levels of NO_3^- present in the plants grown on FYM amended soil is that the nitrate levels within the plant can be moderated by B. The limiting step in conversion of NO_3^- to plant useable forms, is through nitrate reductase (NR) activity. It appears that B is needed to increase the activity of this enzyme. In tobacco, there was a direct positive correlation between the foliar levels of NO_3^-, NR activity and B levels, indicating that at higher B levels there were higher levels of NO_3^- (Ruiz et al., 2001).

Magnesium (Mg) levels were significantly higher in *A. thaliana* plants grown on FYM amended soil throughout the experiment, while potassium (K) and calcium (Ca) levels were only significantly higher for the first and third time points.

Mineral levels present in the Palace Leas field trial are presented in Table 3.6. Mg and Ca levels are higher in Plot 2, which is amended with FYM, than in Plot 13, while K levels are higher in Plot 13 (Shiel, personal communication). The higher levels of Mg and Ca present in the soil are reflected in the higher levels of these minerals present in the plant tissues. The presence of higher levels of K in the plants grown on FYM amended soil is interesting, as this is the only cation found to be present at a lower level in Plot 2.

With increased nitrate nutrition, there is often an increased uptake of inorganic cations (such as Ca^{2+}, K^+ and Mg^{2+}) to counterbalance the increase in organic anions such as malate (Kirkby and Knight, 1977). In a study comparing different fertilisers Turan and Sevimli (2005) showed that, for a given dose of N, the levels of Mg and Ca were not affected by treatment type. When potassium nitrate was used as the primary source of inorganic nitrogen, the levels of K were increased, which was expected by the authors, due to the added availability of K from the fertiliser. It is possible that in the results presented here, the increased levels of these minerals in plants grown on FYM amended soils is driven by an increased amount of bioavailable N in the soil.

The two ratios reported in this study are suggested to be important by Chaboussou (1985). In his book "Healthy Crops; a New Agricultural Revolution" he suggests that the relationship between K and Ca or Mg are important, as the balance between "these cationic elements has the greatest effect on protein synthesis". In the results presented here, *A. thaliana* grown in soil treated with FYM had the higher K/Ca and K/Mg ratios. Crane and Steward (1962) originally hypothesised that the K/Ca ratio, when high, was an indicator of high protein synthesis, while when it was low indicated that there was an increased level of proteolysis. Chaboussou also provides evidence that plants are better able to control diseases such as scale rot in fruit trees if the K/Ca ratio is adjusted in the favour of K (Chaboussou, 1985). Furthermore, more recent studies have indicated that a high K/Ca ratio provides an indicator of drought tolerance in wheat (*Triticum aestivum* L.) (Majid et al. 2007). It has also been identified as a ratio that can identify fruit quality in apples (Amiri et al., 2008).

The K/Mg ratio can also be used as an indicator of plant health in a similar manner to the K/Ca ratio. However, Chaboussou considered a K/Mg ratio of over 7 (as reported here in plants grown on FYM amended soil) as being a marker of a Mg deficiency in the plant.

The results presented here show that *A. thaliana* grew at a faster rate on soil treated with a long-term liberal application of fertiliser in the form of farmyard manure, than plants grown on soil treated using inorganic fertilisers. However, there was no convincing evidence that FYM amendment could produce higher levels of biomass or increased yield than conventional amendment. Mineral levels in the plot soil are likely to have directly influenced the levels present within the plant tissue. There is some evidence that the increased growth rate seen could be due to higher levels of micronutrients such as boron present in plants grown on FYM amended soils, which allows for increased enzyme activity. There is also evidence that the nitrogen status of plants grown on the two treatments are similar, through examination of the chlorophyll levels. Mineral ratios indicated that plants grown on FYM amended soils were either able to perform higher levels of protein synthesis or were deficient in magnesium.

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Chapter 4. Effects of Soil Management Practice on Protein Expression in *Arabidopsis thaliana* as a Model Plant.

4. 1. Abstract

Analysis of the plant proteome is a powerful tool to identify the effects of changing the environment at a molecular level, as it provides biologically relevant information on how the changes are affecting the plant. To identify how fertiliser amendment affects the proteome of *Arabidopsis thaliana*, soil from two plots of the Palace Leas long term field trial were used. The two plots used were: Plot 2, which receives farmyard manure as fertiliser and Plot 13, which receives inorganic fertiliser. Approximately 500 protein spots were reproducibly detected in extracts from *A. thaliana* plants (28 days old approximately) after 2D gel electrophoresis. Of these, 46 protein spots were identified as being differentially expressed, with 30 protein spots being up-regulated and 16 being down-regulated in plants grown of conventionally amended soil; the highest fold changes observed were a 6.18-fold increase in protein spot 20 and a 5.95-fold decrease in spot number 4. Proteins identified as up-regulated in conventionally grown plants were nitrilase 1, 14-3-3 like protein GF14, Heat Shock Cognate 70kDa Protein 1, and Glutathione-S-Transferases PM24 and ERD13, whilst down-regulated proteins included lipoxygenase and Annexin D6. Heat shock protein, lipoxygenase and glutathione-S-Transferases are all directly implicated in the plant stress response, while the other proteins identified have been shown to have differential regulation under conditions of stress. These results suggest that *A. thaliana* is under increased stress when grown in conventionally treated soil.

4. 2. Introduction

The study of proteomics examines the qualitative, quantitative and functional aspects of proteins avaliable within a cell or organism. Proteins show increased diversity from their original genomic sequence due to alterations such as alternative splicing at the transcriptional level or post-translational modifications, such as phosphorlyation or glycosylation. This means that from the 27,000 predicted genes in *Arabidopsis thaliana*, a total of 36,000 proteins have been either identified or predicted (Kersey et al., 2005). This makes the study of protein expression patterns essential as transcriptomic and genomic studies will not provide a complete overview of what is occurring in the whole organism. Furthermore, proteins are also the agents of cellular reactions, so identification at this level provides a valuable tool in elucidating gene function (Hunter et al., 2002).

Proteomics is a well established tool in animal and medical biology and is starting to become increasingly used in plant biology, although currently the majority of studies are limited to 2-D electrophoresis and MALDI-TOF/nano-LC-MS tandem approaches. Second-generation proteomic techniques, such as gel-less technologies (e.g. Multi-Dimensional Protein Identification Technology), the study of the interactome and the study of post translational modifications are only slowly being adopted by the plant biology community (Jorrin et al., 2007). However, the 2D PAGE methods, coupled with powerful image analysis are sufficiently effective to detect changes in protein expression levels (Mehta et al., 2008).

Proteomic studies are increasingly being used to identify the plant response to many different stresses, both biotic and abiotic (Rossignol et al, 2006; Ferry et al 2011). For example, abiotic stress, such as temperature stress and salt stress have been examined in detail using 2D-PAGE (Aghaei et al, 2008, Hashimoto and Komatsu, 2007). These studies have led to the identification of Heat-Shock Protein (HSP) family members being up-regulated as a non-specific response to any form of abiotic stress. There is a similar up-regulation of proteins involved in the Reactive Oxygen Species pathways (Timperio et al., 2008). These responses are likely to be complementary, with ROS being responsible for the oxidative burst defence mechanism, while HSPs will act to protect plant proteins against this effect. The breakdown of RuBisCo under conditions of drought stress in wheatgrass (*Elymus elongatum* Host) has also been reported (Gazanchian et al., 2007).

Biotic stresses have also been investigated using proteomic techniques. Many studies have focussed on plant-pathogen interactions (Mehta et al, 2008). The role of PR (Pathogen Resistance) proteins are well known and easily identified by proteomic techniques. These include thaumatins, glucanases and peroxidases. Again, there is up-regulation of HSPs and ROS related proteins, as part of a general stress response (Mehta et al., 2008).

Insect herbivory also causes changes in the plant proteome. Elicitation of *Nicotiana attenuata* by fatty acids from *Manduca sexta* caused up-regulation in proteins involved in primary and secondary metabolism and control of transcription and translation. Photosynthetic-related proteins, including RuBisCo activase, were down-regulated (Giri et al., 2006). In spruce bark (*Picea sitchensis*), small HSPs were up-regulated in response to weevil herbivory, possibly to mediate damage caused by oxidative burst, in a similar manner to its role in abiotic and pathogenic stress response. Again, proteins involved in secondary metabolism were up-regulated (Lippert et al., 2007).

Due to these, and other studies, techniques to investigate the proteome are at the forefront of agricultural research. As an example, 2D-PAGE has been used to show that the insertion of transgenes have no unintended effects on the proteome of *Arabidopsis thaliana* seed and tomato (*Solanum lycopersicum*) seedlings (Ruebelt et al., 2006, Corpillo et al., 2004). The technology can also identify when there are unintended effects, such as the addition of Cry1Ab, a potent anti-insecticidal protein derived from a bacterium (*Bacillus thuringiensis*) in maize (*Zea mays*). This caused differential regulation of primary metabolism proteins, as well as the unique expression of two dehydrogenase genes in the transgenic maize (Albo et al., 2007).

Identification of proteins as candidates for developing plants with increased stress tolerance is also possible with proteomic techniques by examining differential expression between tolerant and susceptible cultivars. This allows the identification of stress markers that can then be used in breeding programmes using a ‘reverse genetics’ approach (Salekdeh and Komatsu, 2007).

Since the 1950s, agriculture has gone through the so-called 'Green Revolution'. Global cereal yields have doubled in the last 35 years. This increase in yield has been caused by the application of synthetic fertilisers and pesticides, usage of which has also increased significantly (Tilman et al., 2002). However, it is believed that this increased use of fertiliser and pesticides, along with other farming practices, such as monoculture and removal of field margins, has damaged the natural environment (Carson, 1962 Krebs et al., 1999). In recent years, the organic farming movement has become increasingly popular, due to public fears about chemical, especially pesticide, residues in the food products, along with negative perceptions about genetically modified foods. The increase in the organic market sector has been accompanied by an increase in the number of studies examining the ecological impact of organic management practices (Bengtsson et al., 2005, Hole et al., 2005). Yields have also been examined (Badgley et al., 2007), as has the metabolome of crops (Maggio et al., 2008). However, very few comparative studies have focussed on differential gene expression within plants grown using organic versus conventional management practices. Recently a study on the proteomics of potato tubers grown organically has been published. Comparisons between these tubers and ones grown conventionally showed quantitative differences in 160 protein spots, of which 143 were up-regulated. Of these up-regulated proteins, a number were involved in protein degradation, along with defence related enzymes, indicating that organic crops are growing under conditions of increased stress (Lehesranta et al., 2007).

In wheat, microarray analysis showed differential expression of genes. A total of 474 genes were up-regulated in plants amended with Farmyard manure (FYM), while a further 190 genes showed higher expression in treatments where either no or very low levels of nitrogen were applied. Of the genes up-regulated in FYM amended plots, many were identified as being involved in nitrogen metabolism and protein, lignin and flavanoid biosysnthesis (Lu et al., 2005). Synthesis of secondary metabolites, such as lignins and flavanoids, indicates increased stress.

With the increased evidence that soil management practice affects differential gene expression in plants,, experiments were designed to study the effects of different fertiliser regimes on differential gene expression at the proteome level. Since proteins are the final products of gene expression, any differential expression can be considered as a ‘real’ change. Two-dimensional gel electrophoresis, coupled with image analysis and a protein identification method such as Matrix Assisted Laser Desorption and Ionisation Time Of Flight mass spectrometry (MALDI-TOF MS) provides a powerful method for identifying these differences in expression and were thus the methods used in the present study. *Arabidopsis thaliana* was used as the model plant (see chapter 3). The small size and rapid growth means that high number of replicates can be performed in any given experiment. The sequencing of the *A. thaliana* genome, identification of the proteome, together with databases such as MASCOt (used in this experiment), allows rapid and reliable identification of proteins from mass spectrometry data.

Soil from Palace Leas, a long-running experimental plot system at Cockle Park Experimental Farm, Newcastle University, was used as the growing medium in order to investigate the effects of different management practices.

4. 3. Materials and methods.

4. 3. (1) Biological materials

4. 3. (1) (i) Soil collection and processing

Soil samples were collected from the Palace Leas long-term field trial (initiated in 1897) at Cockle Park Experimental Farm, Newcastle University. Samples were taken from two plots in this trial; Plot 2, which is amended with farmyard manure (FYM) and Plot 13, which is amended with inorganic fertilisers (Shiel, personal communication). Plot 2 receives 20t per hectare of farmyard manure (FYM) per year. Plot 13 receives 35kg ha^{-1} nitrogen, 60kg ha^{-1} phosphorus (as P_2O_5) and 67kg ha^{-1} potassium (K_2O). Samples were taken using an auger to a depth of 20cm and from across the the length of the plot. Samples were taken within 2 weeks of treatment application. The soil cores were sieved through a 2mm sieve to remove biological material and to achieve greater uniformity. Samples were stored at 4ºC until required.

4. 3. (1) (ii) Plant growth conditions

Arabidopsis thaliana (ecotype C24) seeds were placed on wetted filter paper and vernalised at 4ºC for 48 hours. Soil was prepared for potting by mixing with perlite to a final concentration of 30% so as allow adequate drainage. 7.5cm pots were filled and between 2 and 5 seeds were placed onto the surface of the soil. To allow germination, humidity was increased using catering film taped to the pots.

All plants were grown in a controlled environment room with a 16/8hr day/night light cycle and a 22/20ºC day/night temperature cycle. Illumination averaged at

150µmol s^{-1} m^{-2}. Sufficient water was applied throughout the course of the growing period so as to keep the soil moist. Plants were sampled at growth stage 1.12 (Boyes et al 2001), approximately 28 days after planting but before the emergence of the inflorescence spike.

4. 3. (2) Experimental methods

4. 3. (2) (i) Protein extraction and sample preparation

Protein extraction followed a similar method to that of Damerval et al. (1986). Plant tissue was ground into a fine powder under liquid nitrogen and transferred to an oakridge tube. Ice cold acetone containing 10% trichloroacetic acid and 0.07% 2-mercaptoethanol was added at a ratio of 20:1 (w/v) and left to precipitate at -20ºC for 2hr. The samples were centrifuged at 14,000g for 15 minutes at 4ºC and the supernatant removed. 20ml acetone containing 0.07% 2-mecaptoethanol was then added as a wash step. Each sample was vortexed for at least 1 minute before being precipitated at -20ºC for 1 h. The samples were again centrifuged at 14,000g for 15 minutes at 4ºC and the supernatant removed. Each sample was dried under vacuum to remove all residual traces of acetone. The vacuum dried material was resuspended in 8M urea, 4%CHAPS, 20mM DTT, 0.5% 3-10NL IPG Buffer (GE Healthcare) at a concentration of 1g/ml and the samples were centrifuged at 14,000g for 10 minutes at 4ºC. The supernatant was collected and stored at -80ºC.

Samples were quantified using a 2D protein Quant Kit (GE Healthcare, UK). Protein yields were between 3 and 5µg/µl.

4. 3. (2) (ii) 2D Electrophoresis

4. 3. (2) (ii) (i) Iso-electric focusing (IEF)

500µg of protein (for each sample) was then "cleaned" using the Clean Up kit provided by GE Healthcare. Briefly, each sample was precipitated using a precipitant in combination with a co-preciptant and then washed using an acetone based agent. All protein samples were resuspended in 340µl of DeStreak Rehydration buffer (GE Healthcare, UK). Samples were loaded onto 18cm 3-10NL IPG strips (GE Healthcare, UK) by active rehydration. 50mA was applied to each strip for 12 hours before IEF. IEF was carried out as follows: 500V step and hold for 0.5kVh, 1000V gradient for 0.8kVh, 8000v gradient for

13.5kVh and 8000v step and hold for 21.2kVh. After IEF each strip was equilibrated in equilibration buffer (6M urea, 75mM tris HCl, 30% glycerol, 2% SDS). Each strip was first equilibrated in buffer containing 50mM DTT for 15 minutes, followed by a further 15-minute equilibration in buffer containing 100mM IAA. Strips were then washed briefly with distilled water before being run on the second dimension step.

4. 3. (2) (ii) (ii) Second dimension gel electrophoresis

All second dimension gels were prepared using the Laemmli system (Laemmli, 1970). Gels were run for 24h at 1.5-2W per gel in the Ettan Six system (GE Healthcare).

4. 3. (2) (iii) Staining

All gels were fixed in fixing solution (40% methanol, 10% acetic acid) for 30 minutes before being stained with Colloidal Coomassie Blue stain (Neuhoff et al., 1988). Gels were stained overnight before being destained with several rinses of dH_2O.

4. 3. (2) (iv) Image analysis

All gels were scanned at 8bit and 300dpi resolution and saved as TIFF file format.

Progenesis SameSpots software (Non-linear Dynamics, Newcastle upon Tyne, UK) was used to analyse the gels. Spots were selected manually. Progenesis was used to generate a list of spots with a fold change greater than ±1.5 fold.

4. 3. (2) (v) Protein identification

4. 3. (2) (v) (i) In-gel digestion

Spots selected by Progenesis were manually punched from a gel and then destained using dH_2O. The digestion protocol was modified from Shevchenko et al. (1996). Briefly, each sample was completely destained using 50mM ammonium hydrogen carbonate and acetonitrile at a 1:1 mix for 15 minutes before being dehydrated using acetonitrile. Samples were rehydrated using 50mM NH_4HCO_3 for 5 minutes before addition of an equal volume of acetonitrile. All samples were dehydrated before being air-dried. 10mM DTT in

25mM NH_4HCO_3 was added to the samples which were incubated for 20 minutes at 56ºC. The liquid was removed from the gel pieces and an equal amount of 55mM IAA in 25mM NH_4HCO_3 was added. The samples were incubated in the dark at room temperature for 15 minutes. All remaining liquid was removed and the samples were washed twice with 50mM NH_4HCO_3 for 15 minutes per wash. The samples were dehydrated with acetonitrile and air dried prior to addition of trypsin. Modified sequencing grade trypsin (Promega) was diluted to 20ng/µl with 25mM NH_4HCO_3 and 2 to 3 µl was added. Samples were incubated at 37ºC overnight. Additional 25mM NH_4HCO_3 was added if needed after 30 minutes. Peptides were extracted from the gel pieces by sonication for 10 minutes. 3µl of 50% acetonitrile, 1% trifluoroacetic acid was added and followed by 10 minutes of sonication. Supernatants were collected and pooled by sample.

4. 3. (2) (v) (ii) MALDI-TOF-MS

Samples were mixed with matrix and spotted onto a steel target plate. The matrix was a saturated solution of 100mg α-hydrocinnamic acid, 33% acetonitrile, 66% 0.1% TFA. The matrix solution was sonicated for 10 minutes after addition of the acetonitrile and for a further 10 minutes after the addition of TFA. The solution was centrifuged at 13,000g for 1 minute at room temperature. Samples were mixed in a 1:1 ratio with the matrix solution. 1µl was spotted onto the target plate and allowed to airdry.

The samples were then analysed using MALDI-TOF-MS (Ultraflex 2, Bruker Daltonics). The equipment was calibrated against a set of peptide standards. A minimum of 1000 hits were used for the calibration. A size range of 400-5000Da was the calibrated size range. Each spot was examined manually with the laser power between 30 and 50%. Each spot had a minimum of 1000 hits recorded. Maximum intensities were in the $1x10^5$ppm range. Peak masses were calculated in the Flex-Analysis software (Bruker Daltonics), using a supplied script. Peaks in the mass range 500-4000Da only were identified.

4. 3. (2) (v) (iii) MASCOT searches

The generated peak list for each sample was queried against the Swiss-Prot database using the MASCOT search engine (Perkins et al., 1999). The *Arabidopsis thaliana* database was queried using 2 missed cleavages, carbamidomethylation (C) and oxidation (M) as modifications. Peptide tolerance was set to ±1.2Da.

Protein scores, intensity coverage and sequence coverage were recorded in Biotools (Bruker).

4. 4. Results

The proteomic expression patterns of *Arabidopsis thaliana* were examined from plants at growth stage 1.12 (28 days approximately, Boyes et al. 2001) grown on soil amended with either FYM (Plot 2) or conventionally amended soil (Plot 13). There were a total of 498 protein spots common between the two treatments. Of these protein spots, 46 were identified as being differentially regulated (Figure 4.1, Table 4.2).

Sixteen protein spots were down-regulated in the conventionally grown plants, while the remaining 30 protein spots were up-regulated. The highest fold changes seen were a 5.93 fold decrease in spot 4 and a 6.18 fold increase in spot 20. The majority of changes were between 1.5 and 3 fold changes, indicating there is 50% to 200% variation in the amount of protein present. From these differentially expressed protein spots, 7 proteins were identified through peptide mass fingerprints (PMFs) generated from MALDI-TOF MS.

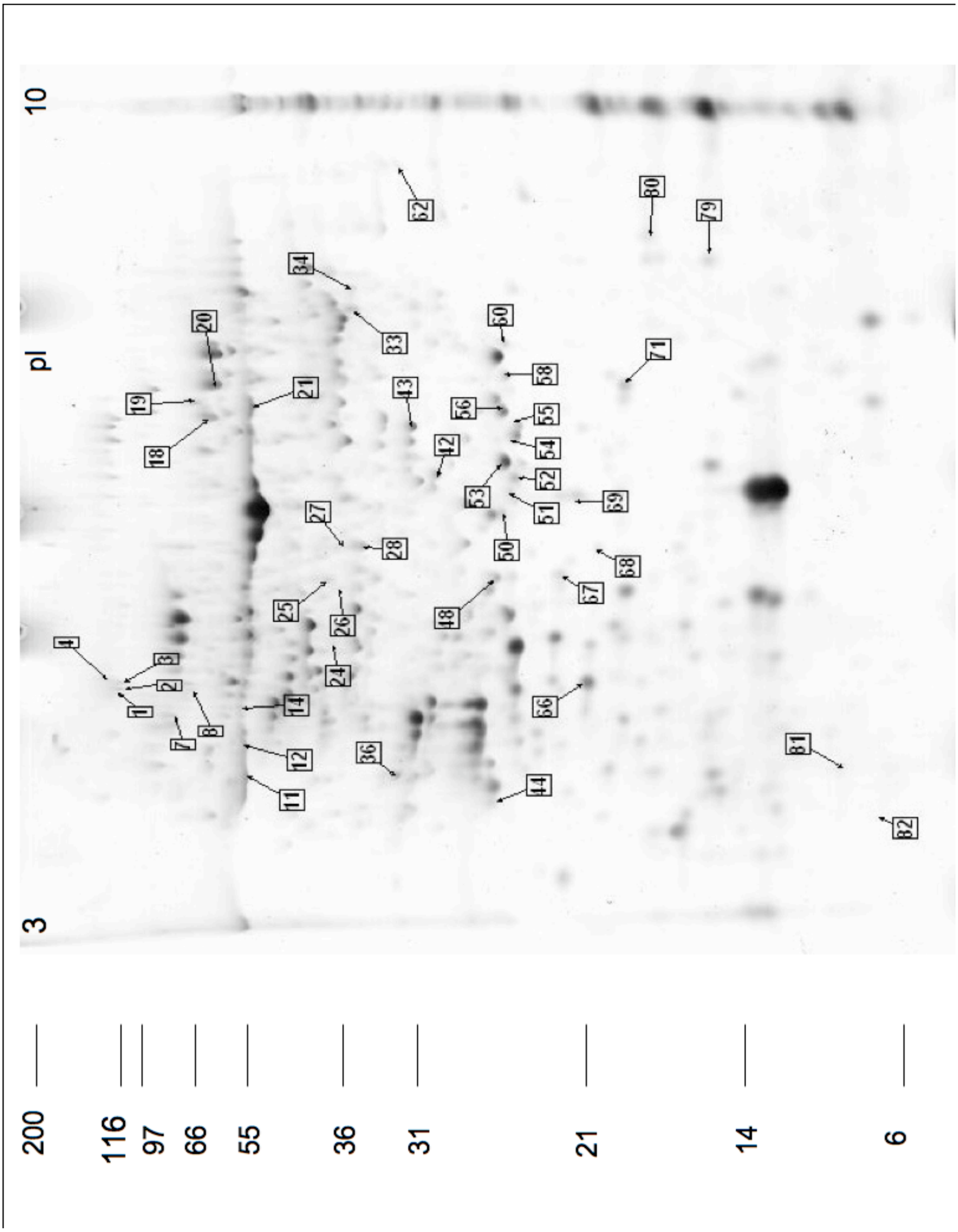


Figure 4.1: Total protein expression profile of *A. thaliana* vegetative tissues using 2-Dimensional gel electrophoresis. Spot numbers denote differentially expressed protein spots in plants grown on soil from Plot 2 (FYM amended) compared to those grown on soil from Plot 13 (receiving conventional fertiliser amendments). Size markers are indicated in kDa. (Spot numbers correspond to those in Table 4.2).

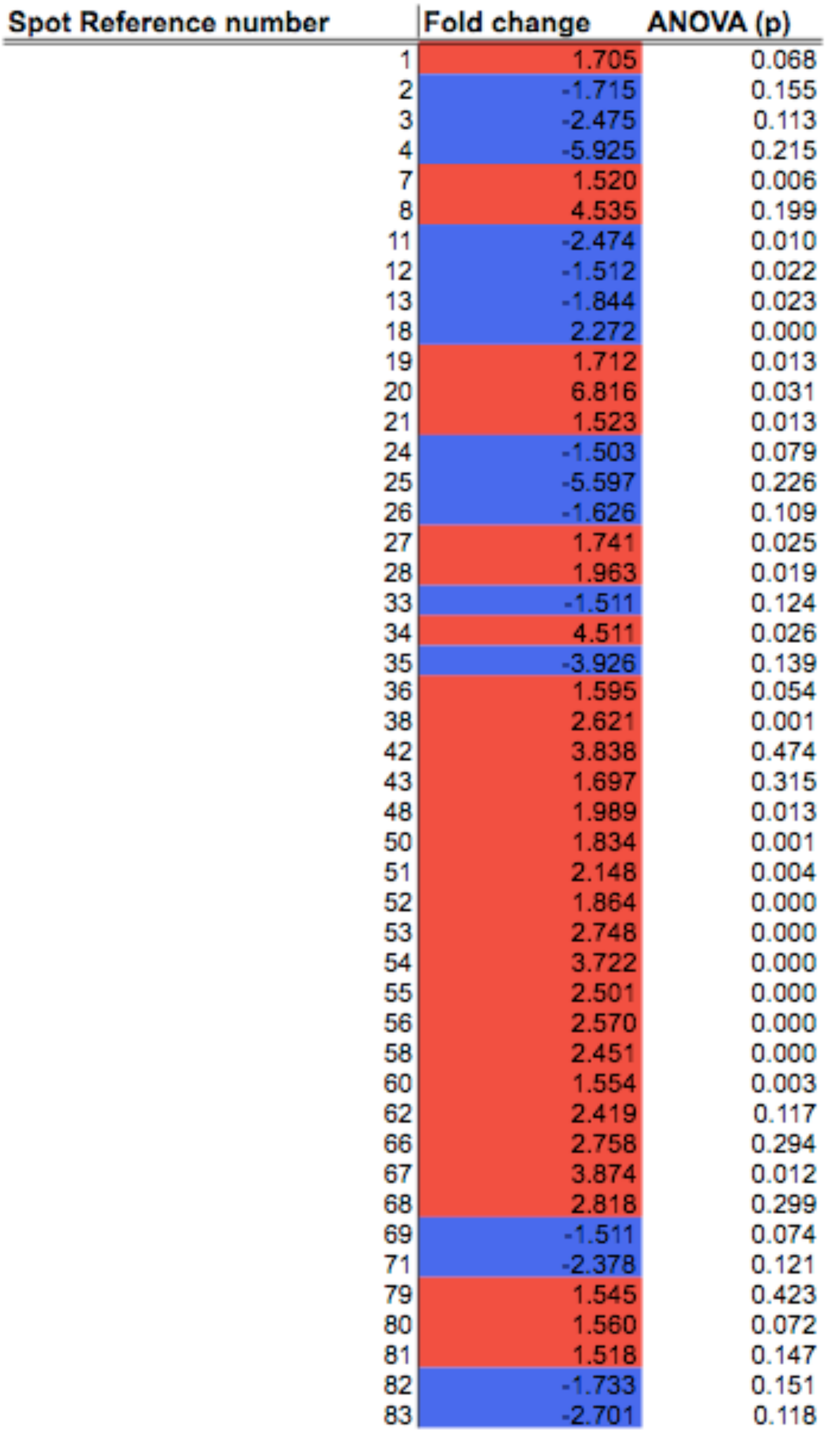
4. 4. (1) Proteins down-regulated in response to fertiliser amendment

Protein spots 2 and 3 were identified as lipoxygenase (LOXC_ARATH). Both of these two spots were down-regulated in *A. thaliana* grown on conventionally amended soil (Plot 13). Spot 33 was identified as Annexin D6 (ANXD6_ARATH) and was also down-regulated in conventionally grown plants. Spots 2 and 3 were both down-regulated in conventionally grown plants (Figures 4.2 and 4.3). However, this down-regulation was not statistically significant when examined with the Student's t-test (spot 2 p=0.51, spot 3 p=0.24). Spot 27 was also down-regulated in conventionally grown plants (Figure 4.4), but again, this down-regulation was not statistically significant (p=0.43). (Table 4.1)

Table 4.1: Identification of proteins by MALDI-TOF and MASCOT. Score values are from MASCOT. Sequence coverage indicates the amount of theoretical sequence predicted by the peptide mass fingerprints. Molecular weight is in kDa. Both molecular weight and pI are the theoretical values.

Spot	ExPasy ID	score	seq. cov (%)	MW	pI	name
2	LOXC_ARATH	109	23.7	102.6	5.3	Lipoxygenase
3	LOXC_ARATH	68	25.8	102.6	5.3	Lipoxygenase
27	NRL1_ARATH	77.9	46.8	38.5	5.8	Nitrilase 1
33	ANDX6_ARATH	51.2	29.9	36.7	8.7	Annexin D6
38	14310_ARATH	66.3	49.2	29	4.6	14-3-3-like protein GF14
39	PSBO1_ARATH	98.1	49.7	35.3	5.4	Oxygen-evolving enhancer protein 1-1
53	GSTF4_ARATH	130	63.7	24.1	5.9	Glutathione-S-Transferase PM24

Table 4.2: Fold changes and statistical likelihood of all proteins identified as being differentially regulated. Red indicates up-regulation of protein spots in samples from conventionally grown plants (Plot 13), while blue indicates down-regulation of spots in samples from conventionally grown plants.



Spot Reference number	Fold change	ANOVA (p)
1	1.705	0.068
2	-1.715	0.155
3	-2.475	0.113
4	-5.925	0.215
7	1.520	0.006
8	4.535	0.199
11	-2.474	0.010
12	-1.512	0.022
13	-1.844	0.023
18	2.272	0.000
19	1.712	0.013
20	6.816	0.031
21	1.523	0.013
24	-1.503	0.079
25	-5.597	0.226
26	-1.626	0.109
27	1.741	0.025
28	1.963	0.019
33	-1.511	0.124
34	4.511	0.026
35	-3.926	0.139
36	1.595	0.054
38	2.621	0.001
42	3.838	0.474
43	1.697	0.315
48	1.989	0.013
50	1.834	0.001
51	2.148	0.004
52	1.864	0.000
53	2.748	0.000
54	3.722	0.000
55	2.501	0.000
56	2.570	0.000
58	2.451	0.000
60	1.554	0.003
62	2.419	0.117
66	2.758	0.294
67	3.874	0.012
68	2.818	0.299
69	-1.511	0.074
71	-2.378	0.121
79	1.545	0.423
80	1.560	0.072
81	1.518	0.147
82	-1.733	0.151
83	-2.701	0.118

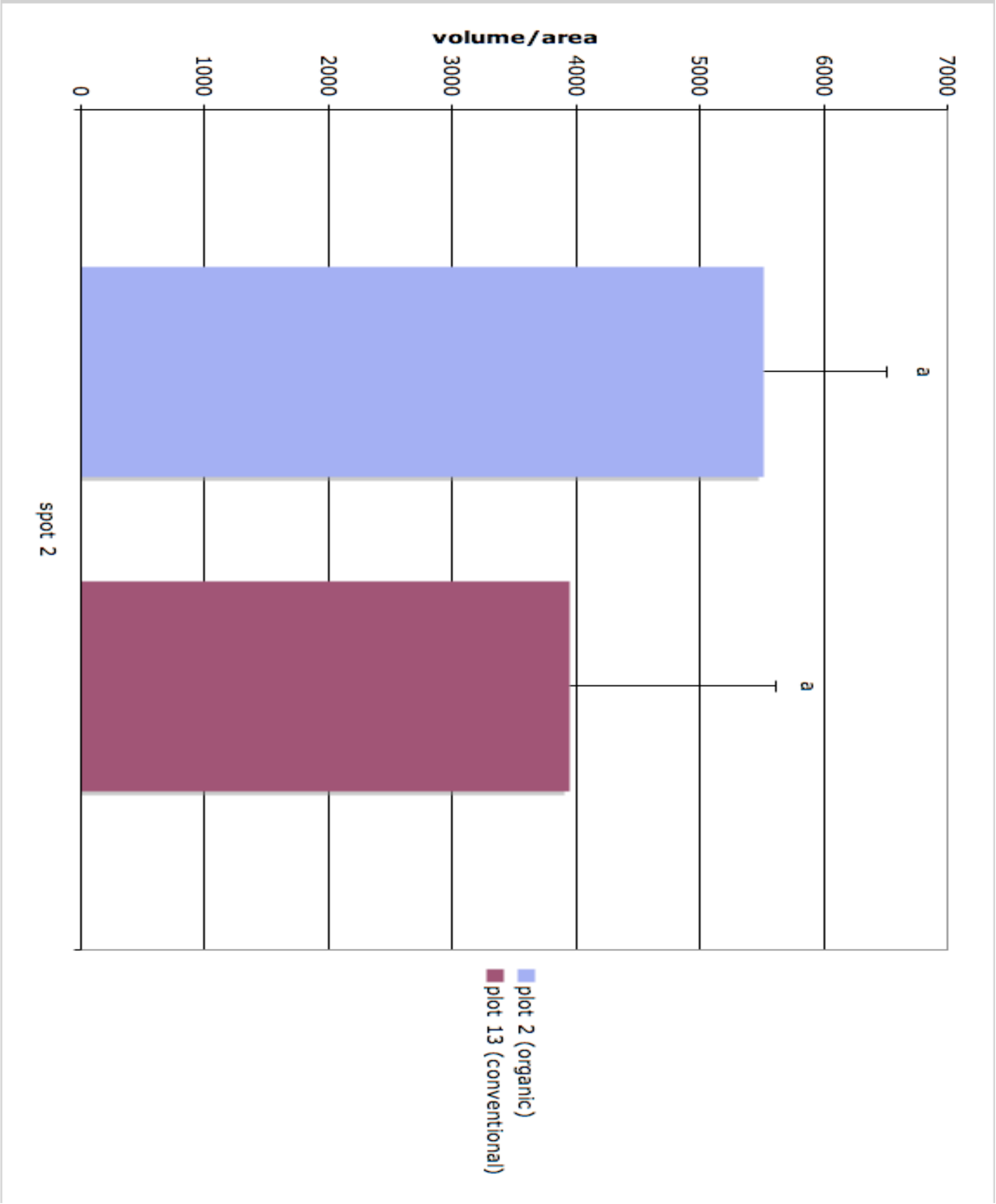


Figure 4.2: Expression levels of protein spot 2 (lipoxygenase) in *A. thaliana* when grown on soil from either Plot 2 (FYM amended) or Plot 13 (conventionally amended). Same letters indicate no statistical significance.

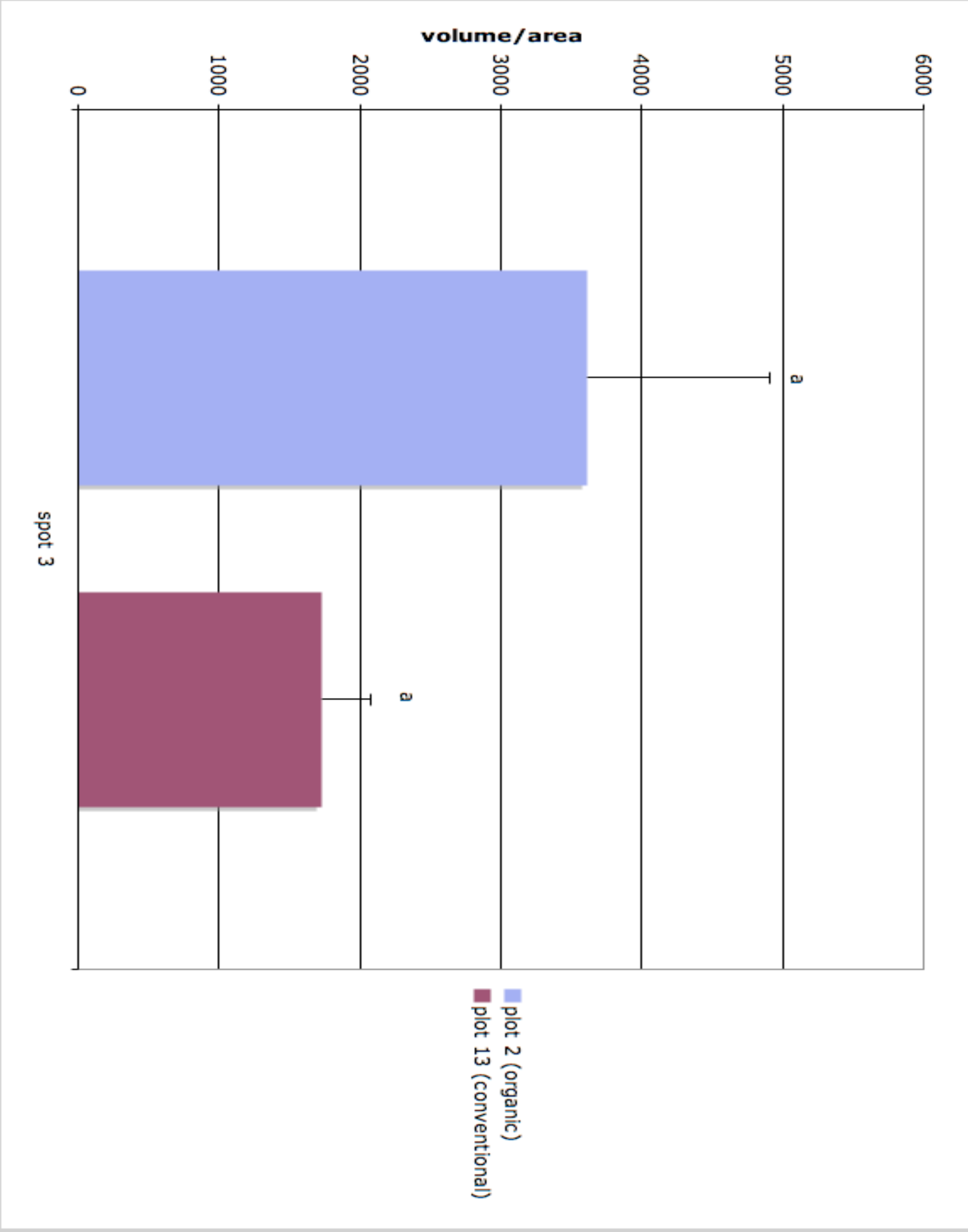


Figure 4.3: Expression levels of protein spot 3 (lipoxygenase) in *A. thaliana* when grown on soil from either Plot 2 (FYM amended) or Plot 13 (conventionally amended). Same letters indicate no significant differences between treatments.

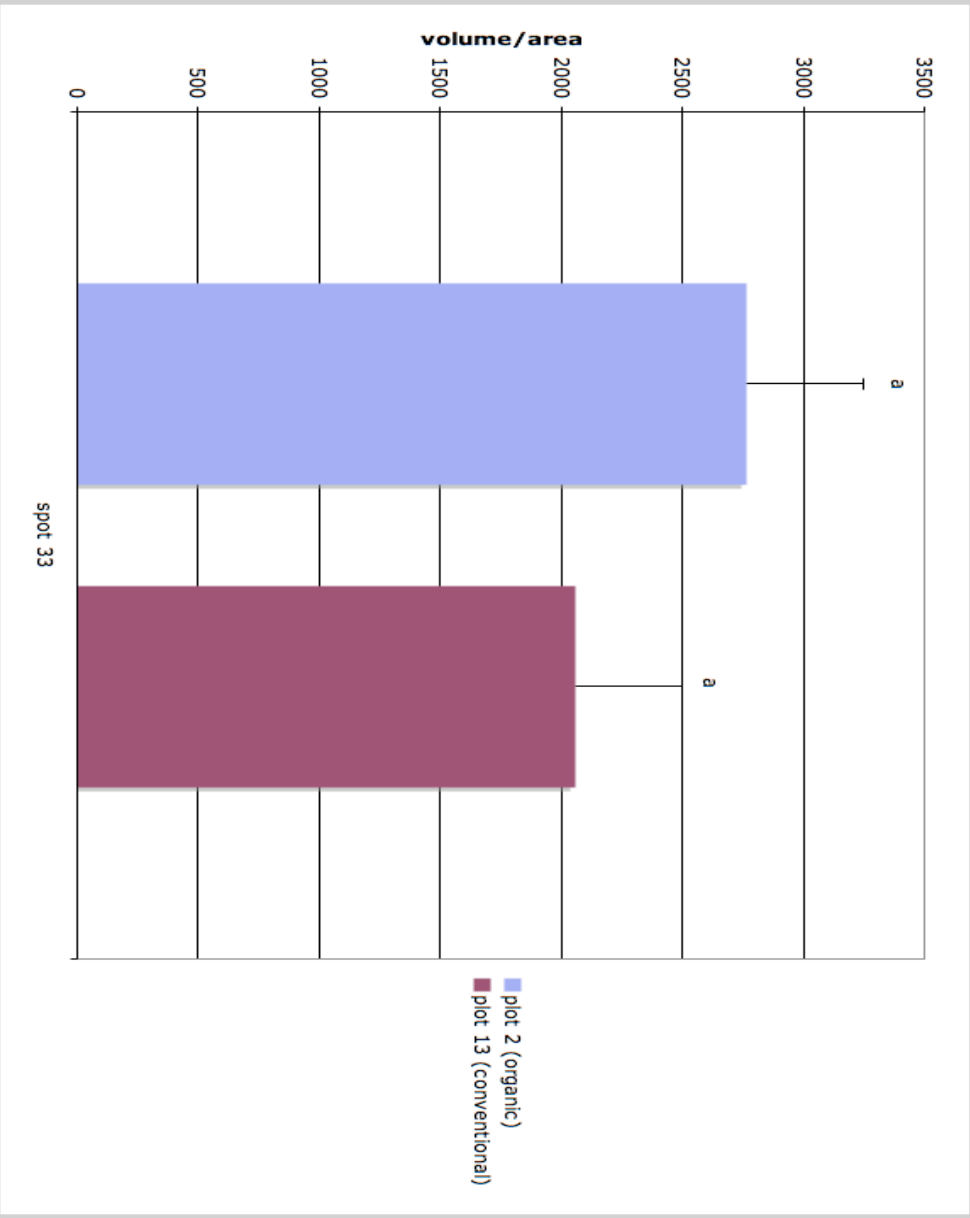


Figure 4.4: Expression levels of protein spot 33 (Annexin D6) in *A. thaliana* when grown on soil from either Plot 2 (FYM amended) or Plot 13 (conventionally amended). Same letters indicate no significant differences between the treatments.

4. 4. (2) Proteins up-regulated in response to fertiliser amendment

Protein spot 27 was identified as nitrilase 1 (NRL1_ARATH) and was shown to be up-regulated in plants grown on soil amended with mineral fertilisers. Similarly protein spot 38, identified as 14-3-3-like protein GF14 epsilon (14310_ARATH) was also found to be up-regulated in conventionally grown plants. Spot 53 was identified as Glutathione-S-Transferase PM24 (GSTF4_ARATH). This protein is up-regulated in conventionally grown *A.*

thaliana.

Thus protein spots 27, 28 and 53 were all found to be up-regulated in conventionally grown plants (Figures 4.5, 4.6, 4.7). Furthermore, all three of these protein spots showed statistical significance (spot 27 p=0.03, spot 38 p=0.01, spot 53 p=0.00). (Table 4.1).

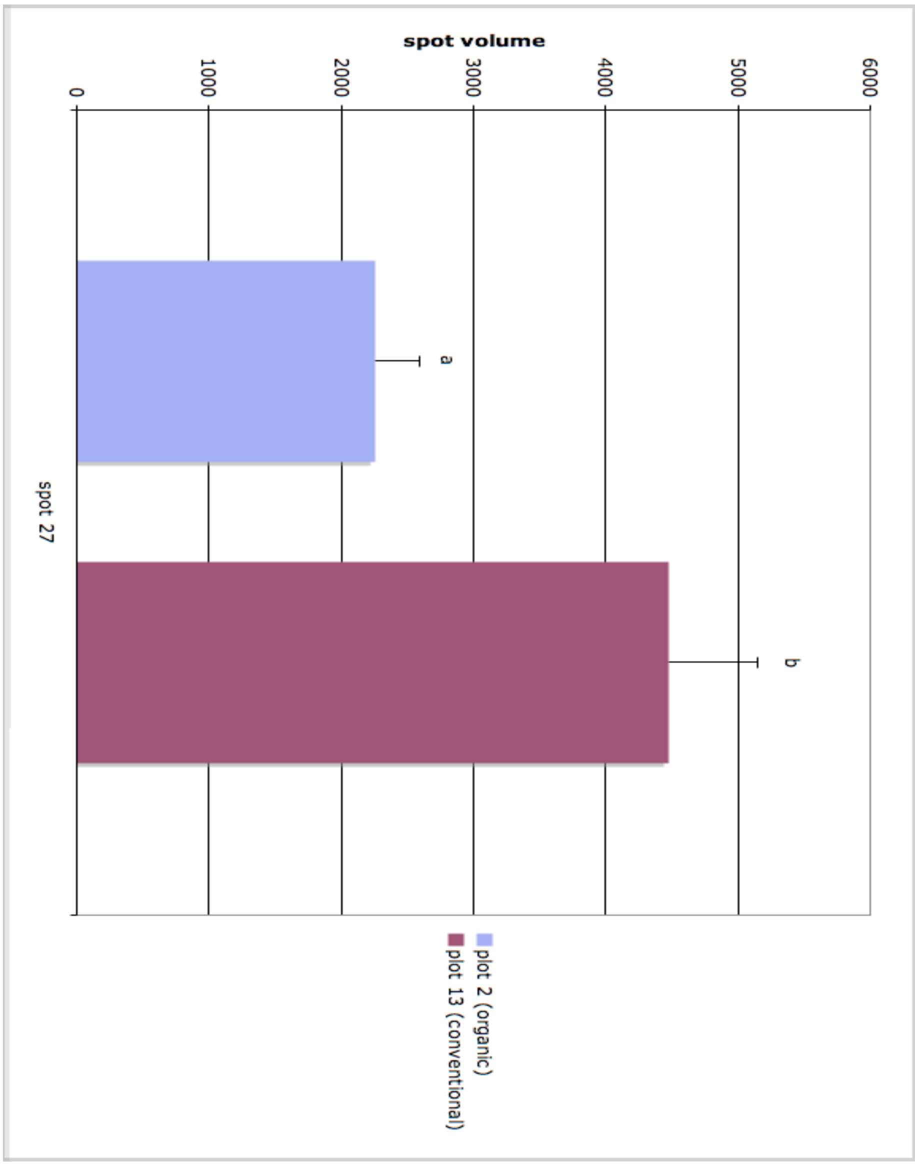


Figure 4.5: Expression levels of protein spot 27 (nitrilase 1) in *A. thaliana* when grown on soil from either Plot 2 (FYM amended) or Plot 13 (conventionally amended). Different letters indicate significant differences between treatments.

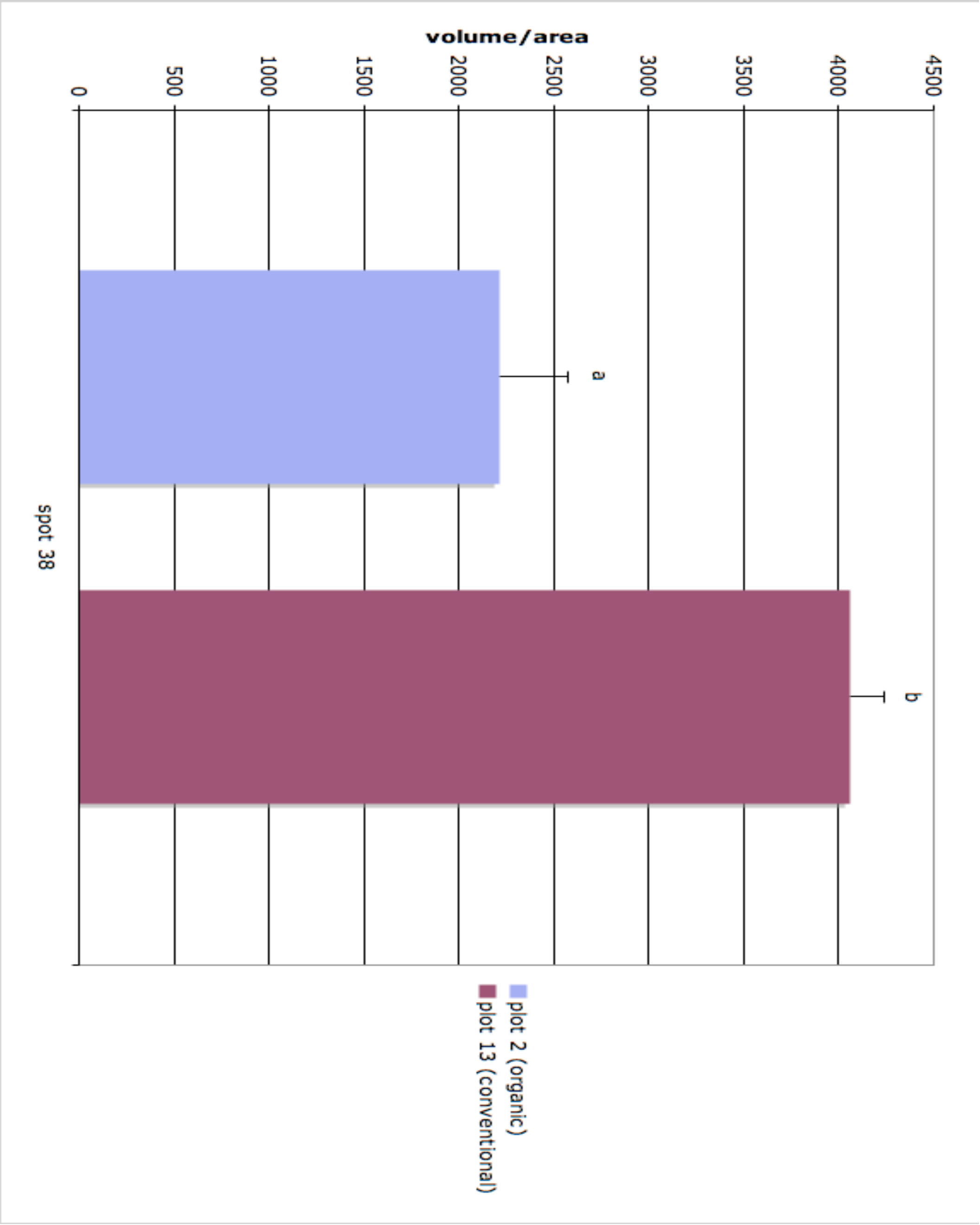


Figure 4.6: Expression levels of protein spot 38 (14-3-3 like protein GF14) in *A. thaliana* when grown on soil from either Plot 2 (FYM amended) or Plot 13 (conventionally amended). Different letters indicate significant differences between treatments.

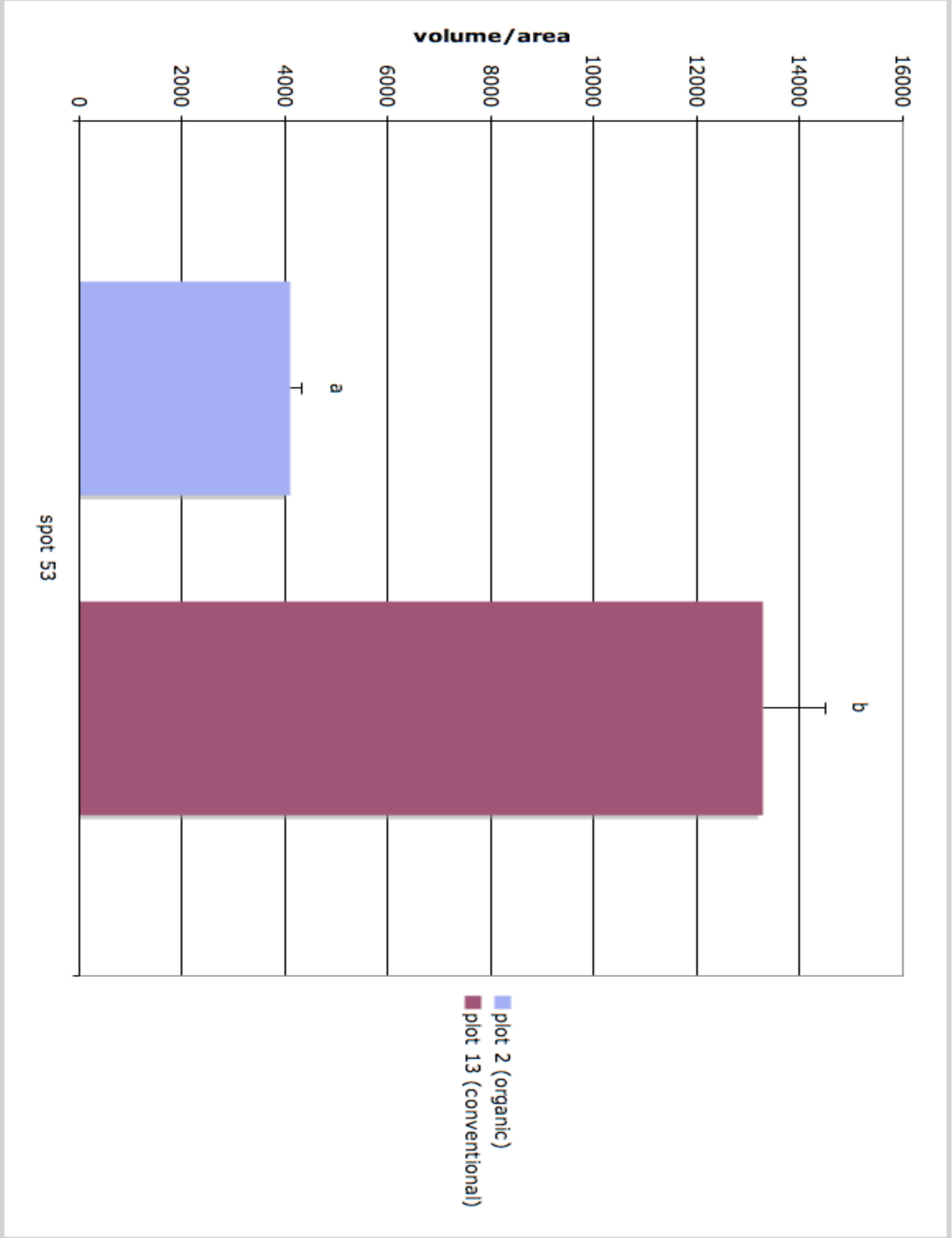


Figure 4.7: Expression levels of protein spot 53 (Glutathione S-transferase PM 24) in *A. thaliana* when grown on soil from either Plot 2 (FYM amended) or Plot 13 (conventionally amended). Different letters indicate significant differences.

4. 4. (3) Identified but not differentially regulated proteins

Protein spot 39 was identified as Oxygen-evolving enhancer protein 1-1 (PSBO1_ARATH). However, this protein was not differentially regulated over the limits set during the analysis (Table 4.1).

4. 4. (4) Putative proteins differentially expressed in response to fertiliser amendment

Two further proteins were identified by MALDI-TOF and were also found to be differentially regulated between the two treatments. However, the practical pI of these proteins do not match their theoretical pI values. Protein spot 8 was identified as a putative Heat Shock cognate 70kDa protein 1 (HSP71_ARATH) and was up-regulated in conventionally amended plants. Spot 56 was identified as a putative glutathione-S-transferase ERD 13 (GSTF3_ARATH). This is also up-regulated in conventionally grown plants. Table 4.3 presents these proteins with both the observed pI and theoretical pI values.

Table 4.3: Identification of proteins identified by MALDI-TOF MS and MASCOT that have mis-matched observed and theoretical pI values. Molecular weight is in kDa.

spot	ExPasy ID	Score	Seq. Cov. (%)	MW	obs. pI	Theor. pI	Name
8	HSP71_ARATH	74.2	30.6	71.7	3.7	4.9	Heat Shock cognate 70kDa protein 1
56	GSTF3_ARATH	97	60.4	24.2	6.6	5.5	Glutathione-S-transferase ERD 13

4. 5. Discussion

The present study investigated the effects of soil management practices on differential protein expression of *Arabidopsis thaliana*. A total of 498 protein spots were expressed commonly between the two treatments but only 46 spots were differentially regulated above a 1.5 fold limit. Of these 46 protein spots, a total of seven were identified by MALDI-TOF MS, while a further two were identified, but did not show a match between the observed pI and the theoretical pI. Interestingly, of those proteins identified several are involved in stress response (GSTF3; GSTF4; HSP71; LOXC). Others are known to be involved in hormone metabolism (NRL1), photosynthesis (PSBO1) and cell signalling (ANXD6; 14310).

4. 5. (1) Stress Related Proteins

Glutathione-S-Transferases F3 and F4 (GSTF3_ARATH, GSTF4_ARATH) and Heat Shock Protein 71 (HSP71_ARATH) are up-regulated in conventionally grown plants. Heat Shock proteins are often seen as a generic marker for increased stress within a plant (Timperio et al., 2008). GSTs are also involved in stress regulation, where they act to detoxify stress induced metabolites such as lipid peroxides, which are produced as a consequence of the oxidative burst mechanism (Ferry and Gatehouse, 2009). They are also involved in the response to auxins and the detoxification of xenobiotics (Marr, 1996). Together, these results indicate that *A. thaliana* grown on soil amended with mineral fertilisers (conventionally amended) from the Palace Leas field trial is under higher levels of stress than the plants grown on the soil amended with farmyard manure (organically amended). This finding is contrary to what would have been expected since plants grown under organic conditions are generally reported as being under higher levels of stress (Lehesranta et al., 2007)

Lipoxygenases (LOXC_ARATH) also play a major role in the production of Reactive Oxygen Species (ROS) when a plant comes under stress, both abiotic and biotic. Lipid membrane molecules are degraded and then peroxidised by lipoxygenase. This production of peroxidised membrane lipids then becomes the major source for the production of ROS produced during the oxidative burst response (Bhattacharjee, 2005). Both spots identified as lipoxygenase were down-regulated in conventionally grown plants, indicating that, contrary to the up-regulation of GSTs and HSP, the plants were under less stress requiring ROS than the organically grown plants. However, it is possible that at the time of sampling, the plants had recently undergone stress and were in the process of repair, hence the down-regulation of proteins involved in the production of ROS.

4. 5. (2) Potential Role of other Differentially Expressed Proteins

Nitrilase 1 (NRL1_ARATH) converts indole-3-acetonitrile into indole-3-acetic acid (IAA), the major auxin phytohormone. Increased production of Nitrilase 1 in conventionally grown plants would indicate that these plants have higher levels of IAA. As the first identified phytohormone, the role of auxins in plant growth regulation are well known (Went and Thimann, 1937)

There is evidence that nitrilase in *A. thaliana* is up-regulated under stress, at least at the transcriptional level. When under stress from high levels of salinity, *A. thaliana* showed increased levels of nitrilase 1 transcripts, indicating that IAA levels might increase in response to salinity stress (Bao and Li, 2002). In the study presented here, nitrilase 1 is up-regulated in conventionally grown plants and so is possibly another indicator that the plant is under stress. However, there is also evidence that under oxidising conditions nitrilase 1 is thiolated *in vitro* (Dixon et al., 2005). It is possible that this modification, rather than activating the enzyme, is actually inactivating it, as one of the target amino acids has a catalytic role (Pace and Brenner, 2001).

While Oxygen-evolving enhancer protein 1-1 (PSBO1_ARATH) was not identified as being differentially regulated over the cut-off values set during the course of this study, it is worth noting that photosynthetic related proteins are often seen to be differentially regulated under stress conditions, often downwards as resources are diverted to surviving the stress (Veeranagamallaiah et al., 2008).

Annexin D6 (ANDX6_ARATH) is part of the *A. thaliana* family of annexins, ubiquitous Ca^{2+} binding proteins. Annexins are multifunctional proteins that are capable of Ca^{2+} dependent or independent membrane binding, actin binding and regulation of ROS and the cytosolic levels of Ca^{2+} (Mortimer et al., 2008). Under conditions of stress, annexin expression is often increased, along with a co-localisation to the plasma membrane. For example when conditions of salinity stress were imposed, levels of annexin (AnnAt1) in *A. thaliana* were increased and there was localisation to the plasma membrane, indicating that annexin in this case is playing a role in Ca^{2+} mediated signalling (Lee et al., 2004). However, as these stress conditions will also cause the production of ROS, there is a possibility that this input mediates the membrane localisation (Mortimer et al., 2008).

In the present study, Annexin D6 is up-regulated in conventionally grown plants. This finding is in agreement with other studies. For example, under high-temperature stress, dehydration and salinity stress, the mRNA transcripts for this specific annexin is up-regulated by between 10- and 12-fold, while it is down-regulated under low temperature stress (Cantero et al., 2006)

The remaining identified protein belongs to the 14-3-3 group of proteins. This is also a ubiquitous, multigene family present in eukaryotic cells, with a known role in cellular signalling pathways. They are able to associate with the G-Box DNA/protein complex (Wu et al., 1997). Transcripts of genes encoding for 14-3-3 proteins in tomato have been identified as showing differential expression under salinity stress and potassium and iron deficiency. At least one of the genes present responds with up-regulation to all three forms of stress, while other genes within the family also show stress induced up-regulation. It is likely that these genes are acting to regulate the response through the G-box interaction and also interactions with plasma membrane H^+ ATPases, which are implicated in stress ion homeostasis (Xu and Shi, 2006). 14-3-3 proteins also appear to be involved in the regulation of ROS. When tobacco cell lines were generated with an anti-sense construct of a 14-3-3 protein gene, ROS production on initiation with cryptogein was between 2%-20% of the control (Elmayan et al., 2007)

In the current study 14-3-3 General Regulation Factor 14 (14310_ARATH) was up-regulated in conventionally grown plants. This is one of several 14-3-3 proteins within the *A. thaliana* genome. It is likely that this up-regulation indicates that these plants were under a form of stress.

I. 5. (3) Conclusions

All the proteins identified within the present study have some role in the plant's response to stress or show differential expression under stress conditions. Most of the proteins are up-regulated in the conventionally grown plants, indicating that these plants are under a higher level of stress compared to those plants grown under organic conditions.

To date, there has only been one comparable study, where the effects of organic agricultural practices on potato tuber proteins were examined (Lehesranta et al., 2007). In that particular study, a significant number of the proteins up-regulated in organically grown potatoes were either directly related to defence, such as Kunitz-type enzyme inhibitors, or were involved in mediating the stress response, such as HSPs and chaperonins. The majority of proteins were up-regulated under organic treatment practices. The few that were up-regulated under conventionally managed systems included an HSP and a protease inhibitor, indicating that there was some insect-related stress in these plants, albeit at a lower level than that experienced by the organic tubers. The conclusions drawn by the authors were that the organically grown tubers were under increased levels of stress, caused by the management practices. However, in contrast to the study by Lehesranta et al (2007), the current study shows that *A. thaliana* grown using the conventionally managed soils exhibited indicators that they were under greater levels of stress.

Plants grown on the conventional soil from Palace Leas often grow to a smaller size; the average hay yield produced by Plot 13 is approximately 1500kg ha^{-1} less than the hay yield from Plot 2, over the course of this experiment (Shiel, personal communication). It was shown in Chapter 3 of this thesis that plants grown in soil from the organically treated Plot 2 grew faster than those grown in soil from the conventionally treated Plot 13. The results presented indicate that the Plot 13 soil might cause increased stress in plants grown in it, which results in reduced biomass production. Thus the data obtained in this chapter on the identification of differentially expressed proteins in response to fertilizer amendment support those presented in Chapter 3 on effects of management practice on plant growth.

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Chapter 5. Volatile and glucosinolate production in *Arabidopsis thaliana* influenced by soil fertiliser amendment in response to insect herbivory.

5. 1. Abstract

The plant's secondary metabolism is one of the mechanisms used to protect against insect herbivory, both directly through molecules with toxic properties and indirectly through the attraction of natural enemies of insect pests (i.e. parasitoids/predators) via the release of volatile chemicals. Both direct and indirect chemical defence mechanisms in *Arabidopsis thaliana* were investigated when plants were grown in soil treated with different fertiliser amendment regimes. Soil was taken from two plots in the Palace Leas long term field trial and from Nafferton Farm; Plots 2 and 13, from Palace Leas, receive farmyard manure (FYM) and inorganic fertiliser respectively, whilst soil from Nafferton farm was taken from neighbouring conventional and organic fields. This provided four different experimental treatments, two amended with FYM and two amended with inorganic fertiliser; however, the levels of FYM and minerals differed between the two sites. Three chemically distinct groups of glucosinolates were shown to be present in *A. thaliana* by HPLC/MS: alkyl, alkylthioalkyl and indole glucosides. However there were both qualitative and quantitative differences between plants grown on conventional vs FYM amended soils. Plants grown on FYM amended soils had higher levels of the alkyl glucoside gluconaprin, whilst those on the conventionally treated soil from Palace Leas contained glucoraphanin and glucoerucin; these two alkylthioalkyls were absent in other treatments. Subsequent aphid infestation of these plants resulted in a small increase in the indole glucosides irrespective of the type of amendment (7% and 8% for conventionally and organically grown plants, respectively), although the differences in response to infestation between the treatments were not significant. Aphid infestation also resulted in a decrease in gluconapin (by 16%), although this was more pronounced in plants grown on conventionally amended soils than FYM amended soils. There were also both qualitative and quantitative differences in volatile emissions from plants grown

on the different soil types. Those grown on the conventionally amended soil (Plot 13 from Palace Leas) produced 26 different volatile chemicals compared to 18 from the FYM amended soil (Plot 2). Furthermore, the average levels of volatiles released where higher from the former than the latter. Infestation by the chewing insect, *Pluetalla xylostella*, reduced both the numbers and levels of these volatiles, although the numbers remained higher in the conventionally grown plants compared to the FYM amended plants (10 and 4, respectively) as did the levels (average of 1089ng (±236) and 306ng (±107), respectively). Aphid infestation (sap sucking insect) resulted in similar trends. The conventionally grown plants released 10 different chemicals at an average level of 1073ng (±102), whilst the FYM amended grown plants released 5 different chemicals, at an average level of 252ng (±54). These results indicate that there was little effect of amendment practice on direct defence mechanisms in *A. thaliana*, although indirect defence was significantly altered by amendment practice.

5. 2. Introduction

Plant defence against insect herbivory involves a diverse set of mechanisms. Plants are capable of maintaining a constitutive defence, utilising compounds produced from secondary metabolism. These products are accumulated within the plant, often in specialist organs, providing the plant with the means to defend itself against herbivory throughout its life cycle (Wittstock and Gershenzon, 2002). This static defence contrasts the other mechanism available, active or induced defence, where defensive compounds are synthesised in response to insect or pathogen attack (Gatehouse, 2002). Induced, defence often utilises protein-based toxins, such as proteinase inhibitors, which block the action of insect gut proteinases, alongside secondary metabolism based compounds.

Secondary metabolites have two roles within plant defence. One is a direct role, as anti-feedants or toxins, while the second role is indirect, where the molecules are volatilised and able to attract natural enemies of pest species. Within *Arabidopsis thaliana*, glucosinolates are the main secondary metabolism-based defence molecules; these metabolites are found almost exclusively in the *Brassicaceae* (Brown et al., 2003). Upon herbivory, glucosinolates are hydrolysed into biologically active products via the action of myrosinase; these products include isothiocyanates, thiocyanates and nitriles (Bones and Rossiter, 1996, Ratzka et al., 2002). These compounds are capable of providing protection against generalist herbivores and pathogens, but they also attract certain specialist herbivores, which are capable of metabolising glucosinolates into a safe breakdown product, as exemplified in the diamondback moth (*Plutella xylostella*) (Ratzka et al., 2002). Other insects, such as the turnip sawfly (*Athalia rosae*), the cabbage aphid (*Brevicoryne brassicae*) and the turnip aphid (*Lipaphis erysimi*) are capable of sequestering the glucosinolates from the plant and using them as a form of defence when stressed (Müller et al., 2001, Bridges et al., 2002).

Glucosinolates are one of the plant defence molecules that are constitutively stored in plants. The spatial separation between glucosinolates and myrosinase prevents the generation of toxic compounds within the plant cells. In *A. thaliana* inflorescences, glucosinolates are present in high concentrations in cells between the phloem and endodermis (Koroleva et al., 2000) while myrosinase is localised in phloem parenchyma cells (Andréasson et al., 2001). When tissue is damaged, the glucosinolates come into contact with myrosinase to release biologically active compounds (Wittstock and Gershenzon, 2002). Genes regulating glucosinolate metabolism are differentially regulated on insect herbivory. In a recent study, genes involved in the biosynthesis of indole, aliphatic and benzyl-glucosinolates were all up-regulated in response to feeding by *Plutella xylostella* (Ehlting et al., 2008). Similarly when plants were under attack from specialist (*Brevicoryne brassicae*) and generalist (*Myzus persicae*) aphids, the indole glucosinolate pathway was up-regulated (Kuśnierczyk et al., 2007).

As noted above, on wounding there is release of volatile chemicals. The volatiles released include a wide range of compounds, such as indole and C_{10} and C_{15} terpenoids, along with the 'green leaf' volatiles, for example, alcohols, esters and C_6 aldehydes (Paré and Tumlinson, 1999). The release of these chemicals is both local to the wounding site and also a systemic response, mediated by jasmonate. The green leaf volatiles are able to induce a wounding response (Gatehouse, 2002).

Indirect defence is the ability of the released volatiles to attract predators or parasitoids of the herbivore. When infested with the larvae of *Pieris rapae, Arabidopsis thaliana* produces a mixture of volatiles that attract *Cotesia rubecula*, a parasitoid wasp of *P.rapae* (Van Poecke et al., 2001). The blend of volatiles released under caterpillar herbivory was similar to the blend released when the plants were mechanically wounded. However, there were nitriles and a terpenoid released from the caterpillar infested plants along with a higher level of methyl salicylate. It is likely that the terpenoid (myrcene) is the chemical responsible for attracting *C. rubecula* (Van Poecke et al., 2001).

Different species of herbivore produce different blends of volatiles, for example, in maize (*Zea mays)* stem borer (*Ostrinia nubilalis*) damage led to the plant producing lower levels of volatile emission than from damage caused by *Spodoptera littoralis*, in addition to some unidentified (at the time of publication) highly volatile compounds. In contrast, aphid damage, even at high levels of herbivory, did not cause any changes in the amounts or types of volatiles produced (Turlings et al., 1998). In *A. thaliana*, non-host chewing herbivores were found to cause the production of volatiles similar to those produced by the host herbivore, to strongly attract the specialist parasitoid, *C. rubecula*. This study shows that when leaf-chewing insects infest the plant, the volatile blend is sufficiently similar to attract a specialist parasitoid wasp, regardless of whether the insect is the host or not. The wasp only detects a difference in the volatile blend when the mode of damage is different, for example, when presented with a choice between 'sucking' and 'chewing' insects. Insects that cause very little tissue damage, such as the aphid *Myzus persicae* do not attract this parasitoid (Van Poecke et al., 2003). However, *A. thaliana* does emit a volatile blend under aphid herbivory that can attract an aphid-specific parasitoid, such as *Diaeretella rapae*. This parasitoid wasp is attracted to *A. thaliana* when infested with *M. persicae*, but not to the aphids on their own or by mechanically damaged plants (Girling et al., 2006). This attraction of parasitoid or predator has been identified in many different tri-trophic systems (van Poecke and Dicke, 2004).

It is not clear how organic agriculture affects primary or secondary metabolism of plants. In wheat, only 8 of the 52 metabolites showed significant differences between organically or conventionally grown plants (Zörb et al., 2006). However, the levels of difference seen, while being statistically significant, were not large enough to directly affect the nutritional value of the grains. Potato tubers also appear to show a limited response to cultivation techniques. Only one carbohydrate, fructose, was significantly higher in conventionally grown plants. Total protein was higher in organically grown tubers, while free amino acids were higher in conventionally grown tubers than in tubers grown using organic farming practices (Maggio et al., 2008). However, while there seems to be a very low response in terms of primary metabolism to cultivation technique, this is not the case for secondary metabolism. There is some circumstantial evidence that organically grown plants contain higher levels of secondary metabolites (Worthington, 2001, Brandt and Molgaard, 2001). However, a number of studies show contradictory differences in levels of secondary metabolites. For example, Hakkinen and Torronen (2000) found no consistent changes in the levels of flavonols in strawberries when grown under different amendment regimes, while there were significant increases in the levels of polyphenols and flavonols in plums grown using organic techniques compared to their conventionally grown counterparts (Lombardi-Boccia et al., 2004). There is currently no evidence whether the volatile emissions of plants under insect attack is altered by the method of cultivation. Nor is there much consistent evidence on how defensive secondary metabolites are affected.

The major objectives of the present study were to establish whether long-term farm yard manure amendment alters the volatile emission patterns when under different forms of insect herbivory and how this affects the levels of glucosinolates, the main defensive secondary metabolite in *Arabidopsis thaliana*. The soil used in this experiment came from the Palace Leas experimental plots at Cockle Park Farm, Newcastle University and from Nafferton Farm, Newcastle University. The Palace Leas trial has been running continuously since 1896 as a grazing and hay cutting experiment (Coleman et al., 1987). Two of the plots from this site were used, Plots 2 and 13. Plot 2 has been continuously amended with farmyard manure, while Plot 13 has received a standard amendment of nitrogen, potassium and phosphorus containing synthetic fertiliser. Soil from the recently converted organic fields at Nafferton Farm, Newcastle University, as was soil from adjacent conventional plots were also used.

Arabidopsis thaliana was selected for these experiments. It is one of the model plants used for molecular studies; not only has the genome been fully sequenced, but a range of information at the metabolomic level is also available. While *A. thaliana* usually relies on fast growth to avoid herbivory from insect species, it is also capable of producing a range of anti-feedant secondary metabolites, including glucosinolates.

Two different insect species, *Myzus persicae* and *Plutella xylostella*, were used in these experiments. The hemipteran *M. persicae* was used as an example of a sap sucking insect. *M. persicae*, a major crop pest in the UK, has a proboscis that is able to penetrate plant tissue to suck the sap from the phloem, thereby causing minimal damage to the plant. However, this aphid is often a vector for plant diseases, causing significant crop losses. Larvae of the lepidopteran *P. xylostella*, also known as the diamondback moth, uses a chewing mechanism for feeding. This causes serious damage to plant tissue. *P xylostella* is originally a southern European pest and feeds primarily on plants of the order Brassicales.

5. 3. Methods and materials

5. 3. (a) Biological materials

5. 3. (a) (i) Soil collection

Soil samples were collected from the Palace Leas long-term field trial at Cockle Park Experimental Farm, Newcastle University (Grid Reference: NZ 201 915). Samples came from two plots on this trial, plots 2 and 13. Plot 2 receives 20t ha^{-1} of farmyard manure (FYM) per annum, while plot 13 receives 35kg ha^{-1}, 60kg ha^{-1} and 67kg ha^{-1} of nitrogen, phosphorus and potassium. Nitrogen was delivered as a mixture of ammonium sulphate and sodium nitrate, phosphorus as superphosphate and potassium as potassium oxide. The samples were taken using an auger from across the length of each plot to a depth of 20cm. Samples from Nafferton farm were taken from across the length of a recently converted organic field and the neighbouring conventional field. The organic field received FYM equivalent to 170kg ha^{-1} while the conventional field received 180kg ha^{-1}, 134kg ha^{-1} and 200kg ha^{-1} of nitrogen, phosphorus and potassium, respectively in the forms of ammonium nitrate, and a compound of superphosphate and KCl. All soil samples were sieved through a 2mm mesh to remove plant debris and to achieve homogenization; all soil samples were then stored at 4ºC until required. Soil samples were taken within 2 weeks of these treatments being applied.

5. 3. (a) (ii) Plant growth conditions

Arabidopsis thaliana (ecotype C24) seeds were placed onto moist filter paper and treated at 4ºC for 48 hours to induce vernalization. Soil was mixed with perlite to aid drainage, the final perlite concentration was 30%. Individual pots (7.5cm diameter) were filled with soil , labelled and placed into seed trays. Each pot then received between 2 and 4 seeds. After each pot had been planted, a 'tent' was constructed out of clingfilm to increase local humidity to the required level for germination. The controlled environment room was set for a 16/8h day/night light cycle and a 22/20ºC day/night temperature cycle. Illumination averaged 150μmols^{-1}m^{-2}. Plants were watered every 4 days or as needed to keep the soil moist.

5. 3. (a) (iii) Insect cultures

Myzus persicae (Peach-Potato aphid) were cultured on green bean (*Phaseolus vulgaris*) at 18ºC and 16/8hr day/night light cycle and *Plutella xylostella* (Diamondback moth) on chinese cabbage using the same conditions.

5. 3. (b) Experimental methods

5. 3. (b) (i) Plant herbivory

To measure the levels of glucosinolates, *A. thaliana* was grown to a stage just before the production of an inflorescence. Plants were then either infested with 100 aphids for 72 hours, or left uninfested. On completion of the time period, all aphids were removed and all samples frozen at 80ºC until required.

5. 3. (b) (ii) Extraction of glucosinolates

Frozen tissue was freeze-dried for 24 hours. Samples were then ground into powder. One gramme of tissue was boiled in 9ml 70% methanol for 15 minutes, the volume made up to 10ml using deionised water in a volumetric flask and 4ml of this solution was applied to an ion-exchange column containing

100mg of DEAE Sephadex A-25 (Sigma-Aldrich, Poole, UK). The columns were pre-equilibrated using 3ml 0.5M acetic acid/sodium acetate buffer at pH 5.0 overnight, followed by 3 washes with 3ml of deionised water. After the addition of the sample solution, each column was washed with 5x2ml washes deionised water and 2x2 washes of 0.5M acetic acid/sodium acetate buffer (pH5.0). Samples were desulphated overnight using 0.5ml 2.5mg/ml solution of *Helix pomatia* sulphatase (Sigma Aldrich, Poole, UK) in 0.02M acetic acid.sodium acetate buffer (pH5.0). Each column was then eluted using 3x1ml washes of deionised water (Agerbirk et al., 2001, Wathelet et al., 2004).

5. 3. (b) (iii) HPLC-UV-MS conditions

Glucosinolate concentrations were determined by reverse phase high performance liquid chromatography of enzymatically desulphated glucosinolates according to the ISO 9167-1 procedure, using an Agilent 1100 series chromatograph (Agilent, Palo Alto, CA) fitted with a Zorbax Eclipse XDB-C18 150 x 2.1 mm 3.5 µm column (Agilent); a flow rate of 0.25 mL/min was used and the column temperature was set at 30 °C. The mobile phase consisted of A (0.1% formic acid in milliQ water) and B (0.1 % formic acid in acetonitrile (LC-MS quality, Biosolve, Valkenswaard, Netherlands). The starting composition of 1.9 % of B was held constant for 3 minutes. A linear gradient was then applied to reach 20 % of B in 30 minutes. In 2 minutes, the composition of B was decreased to the initial 1.9 %. This composition was maintained for 10 minutes.

UV detection at 229 nm was used for the quantification. The response factors used were those recommended by the ISO 9167-1 procedure. The recommended factor of 1.05 was used for the unidentified GLs. For identification, the mass spectra were acquired using electrospray ionisation in the positive mode on a Esquire HCT mass spectrometer (Bruker Daltonics®, Billerica, MA, USA). The mass spectra were recorded from 50 to 600 mz. A drying N2 flow of 9 l/min, a drying gas temperature of 365 °C, a nebulizer pressure of 40 psi and capillary voltages of 4500V were used.

5. 3. (b) (iv) Volatile Analysis Conditions

Volatiles were collected from *A. thaliana* plants removed from their pots, and the root ball enclosed in aluminium foil and cotton wool. The plants were then placed on a Teflon® base. A glass dome chamber (A.R.S., 15 cm base-diameter, 15 cm high), previously washed with acetone and n-hexane, was placed over the plant on the Teflon® base. Clean air was pumped into the chamber (1.1 l/min) through Teflon® tubing and pulled out of the chamber (1.0 l/min) though side ports and across traps containing 40 mg SuperQ® (Alltech, Deerfield, IL, USA). Four separate volatile collections were handled daily. Volatiles were collected for each insect treatment over 24 hours. The treatments were: (1) Undamaged plants; (2) Aphid-infested plants ; (3) *Plutella*-infested plants. Plants grown on each soil type were subjected to these insect treatments.

SuperQ filter traps were eluted using 150 µl diethylether, as the internal standard nonyl acetate (400 ng) was added to each sample. Extracts were then analyzed by gas chromatography coupled with flame ionization detection (GC-FID) using a Hewlett-Packard model 6890 series. Aliquots of 1 µl were injected with a splitless injector held at 220°C. The column (15 m x 0.25 mm internal diameter) was maintained at 35°C for 0.5 min before being heated to 180°C at a constant rate of 12°C/min. Quantifications of compounds were identified by comparing their areas with internal standards, using Chemstation software (Agilent Technologies, Palo Alto, CA, USA).

5. 4. Results

5. 4. (a) Glucosinolates Present in *Arabidopsis thaliana*

Several glucosinolate compounds were found to be produced by *A. thaliana* (Table 5.1). These glucosides were pooled according to their chemical structure to constitute three groups, namely the alkyl, the alkylthioalkyl (including a sulphur) and the indole (including an aromatic group) glucosides. However, there were a further two unidentifed glucosinolates numbered 4 and 10. These had retention times of 5.20 and 31.5 minutes respectively.

The first part of this study was designed to investigate the variation in the different types of glucosinolates in *A. thaliana* as a consequence of the crop amendment regime. The results demonstrated that changes in the glucoside composition occurred between *A. thaliana* plants grown in organic soil and compared to those grown in conventional amended soil (Table 5.2). Glucoraphanin and glucoerucin, both belonging to the alkylthioalkyl group, only occurred in plants grown in conventional soil from Palace Leas (Figure 5.1). Levels of the three groups were similar between the alkylthioalkyl and indole groups, while the organically grown plants had much higher levels of the single alkyl glucosinolate present; gluconapin (Figure 5.2).

In the second part of this study the potential impact of aphid herbivory on the glucosinolate composition of the plants was investigated. In plants grown on both soil amendment treatments, the percentage of the indole glucosinolate group increased under aphid herbivory (Figure 5.3). This was accompanied by a decrease in the levels of gluconapin although this was much more pronounced in conventionally amended plants, with a reduction of 16%. However, there were no alkylthioalkyl glucosinolates detected in the non-infested conventionally amended plants in this experiment and so this is likely to have caused a significant part of this observed decrease. The relative levels of individual glucosinolates found in the different treatments is summarised in Table 5.3.

5. 4. (b) Volatile Chemical Numbers and Abundance in *Arabidopsis thaliana*

Volatile chemicals released by *A. thaliana* when grown in differently amended soil and under biotic stress were examined, and the number of different chemicals and the amounts released over a 24 hour period recorded. A total of 26 different volatile chemicals were released during the 24hr sampling period from plants grown in soil from Plot 13, the long-term conventionally amended plot from the Palace Leas field trial, while there were a total of 18 released from plants grown in soil from Plot 2 (Figure 5.4a). The average levels of volatiles released (in relative units, designated as U) were also higher from the conventionally grown plants (2650ng ±215) than the organically grown plants (2399ng ±490) (Figure 5.5a). Unfortunately, due to a lightning strike, the data identifying the individual volatile chemicals and their relative levels was destroyed.

Table 5.1: Global glucosinolate profile identified in *Arabidopsis thaliana* by HPLC/MS. * indicates internal standard. (Data from plants grown on the different soil types were pooled) ATA = alkylthioakyl, A = alkyl, I = indole

	Common name	**chemical name**	**type**	**retention time (min)**	**m/z of M+H**
1	Glucoiberin	3-methylsulfinylpropyl	ATA	2.3	344.36
2	Glucoraphanin	4-methylsulfinylbutyl	ATA	3.5	358.17
3	Sinigrin*	allyl	ATA	4.1	280.33
4	Unknown 1			5.2	
5	Gluconapin	3-butenyl	A	10.8	294.34
6	Glucoerucin	4-methylthiobutyl	ATA	19.8	342.38
7	Glucobrassicin	3-indolylmethyl	I	22.5	369.36
8	Glucohirsutin	8-methylsulfinyloctyl	ATA	25.2	414.43
9	4-methoxy-glucobrassicin	4-methoxy-3-indolylmethyl	I	27.4	399.37
10	Unknown 2			31.5	
11	Neoglucobrassicin	1-methoxy-3-indolylmethyl	I	34.3	399.37

Table 5.2: Relative levels of the glucosinolates present in *Arabidopsis thaliana* when grown on the different soil types (Plots 2 and 13 from Palace Leas, representing FYM and conventional fertiliser amendment, respectively; conventional amended and FYM amended soils from Nafferton farm). All values are in percent. PL = Palace Leas soil, NF = Nafferton Farm soil

	Glucosinolate									
treatments	**1**	**2**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
conventional										
PL	40.45	4.75	5.45	25.18	2.22	14.26	5.05	24.32	9.38	9.40
NF	50.41	0.00	7.24	22.18	0.00	15.04	2.53	30.14	14.41	8.46
mean	45.53	2.37	6.34	23.68	1.11	14.65	3.79	27.23	11.89	8.93
St. dev	7.04	3.36	1.27	2.12	1.57	0.55	1.78	4.11	3.56	0.66
organic										
PL	56.15	0.00	4.40	39.29	0.00	12.25	3.64	24.74	2.22	13.47
NF	48.64	1.54	4.50	32.14	1.16	13.31	5.23	29.73	5.31	7.08
mean	52.40	0.77	4.45	35.71	0.58	12.78	4.44	27.24	3.76	10.27
St. dev	5.31	1.09	0.07	5.05	0.82	0.75	1.13	3.52	2.19	4.52

Table 5.3: Relative levels of the glucosinolates present in *A. thaliana* grown under different fertiliser amendment (Plots 2 and 13 from Palace Leas, representing FYM and conventional fertiliser amendment, respectively; conventional amended and FYM amended soils from Nafferton farm) and when subjected to biotic stress from aphid (*Myzus persicae*). All values in percent.

	Glucosinolate									
treatment	**1**	**2**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**
healthy										
conventional	0.00	0.00	0.00	39.71	0.00	0.36	0.00	19.52	33.06	7.35
organic	36.57	0.55	4.21	25.93	0.65	2.78	3.94	16.19	5.92	3.27
aphid infested										
conventional	30.80	1.95	4.27	16.58	0.91	1.09	2.78	18.47	7.91	6.24
organic	37.43	0.00	3.38	23.04	0.00	6.52	2.78	17.48	3.09	6.29

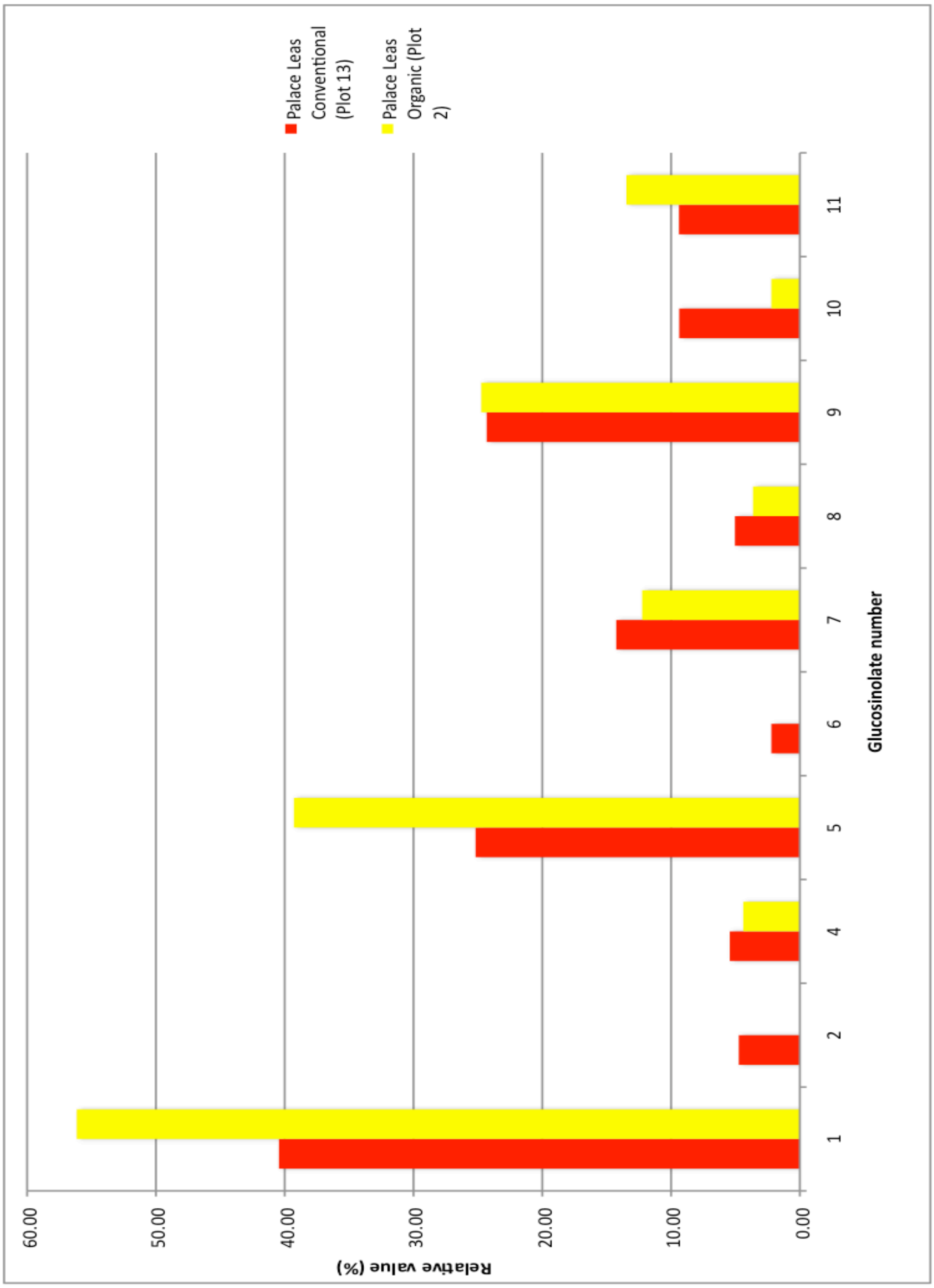

Figure 5.1: Relative levels of individual glucosinolates from *Arabidopsis thaliana* when grown in soil from the FYM amended soil (Plot 2) or conventionally amended soil (Plot 13) at the Palace Leas field trial site. Individual glucosinolates are identified by a number, for ID see Table 5.1 above.

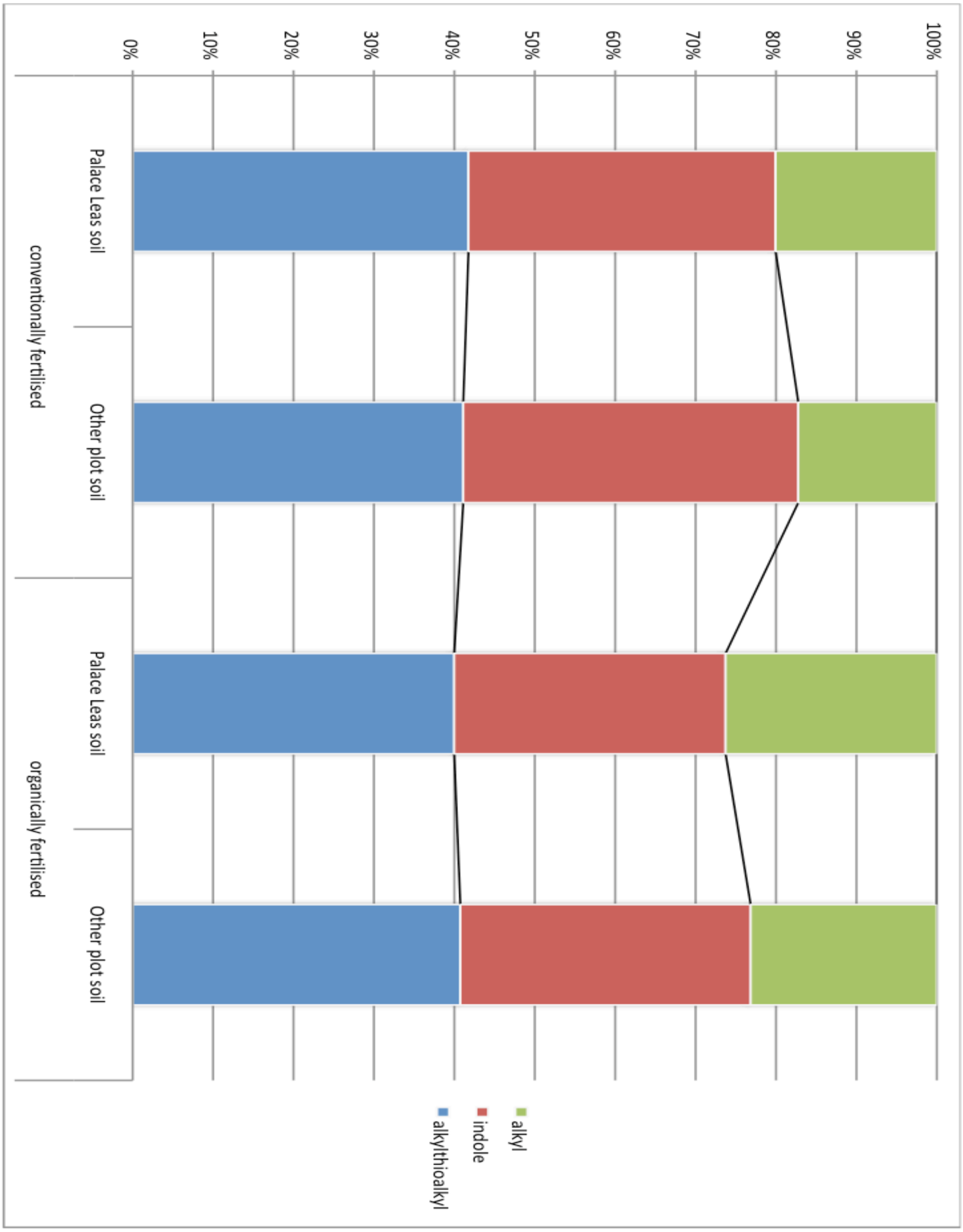

Figure 5.2: Relative levels of the three glucosinolate groups, alkyl, alkylthioalkyl and indole glucosides, present in *A. thaliana* when grown under different soil amendment techniques (Plots 2 and 13 from Palace Leas, representing FYM and conventional fertiliser amendment, respectively; conventional amended and FYM amended soils from Nafferton farm).

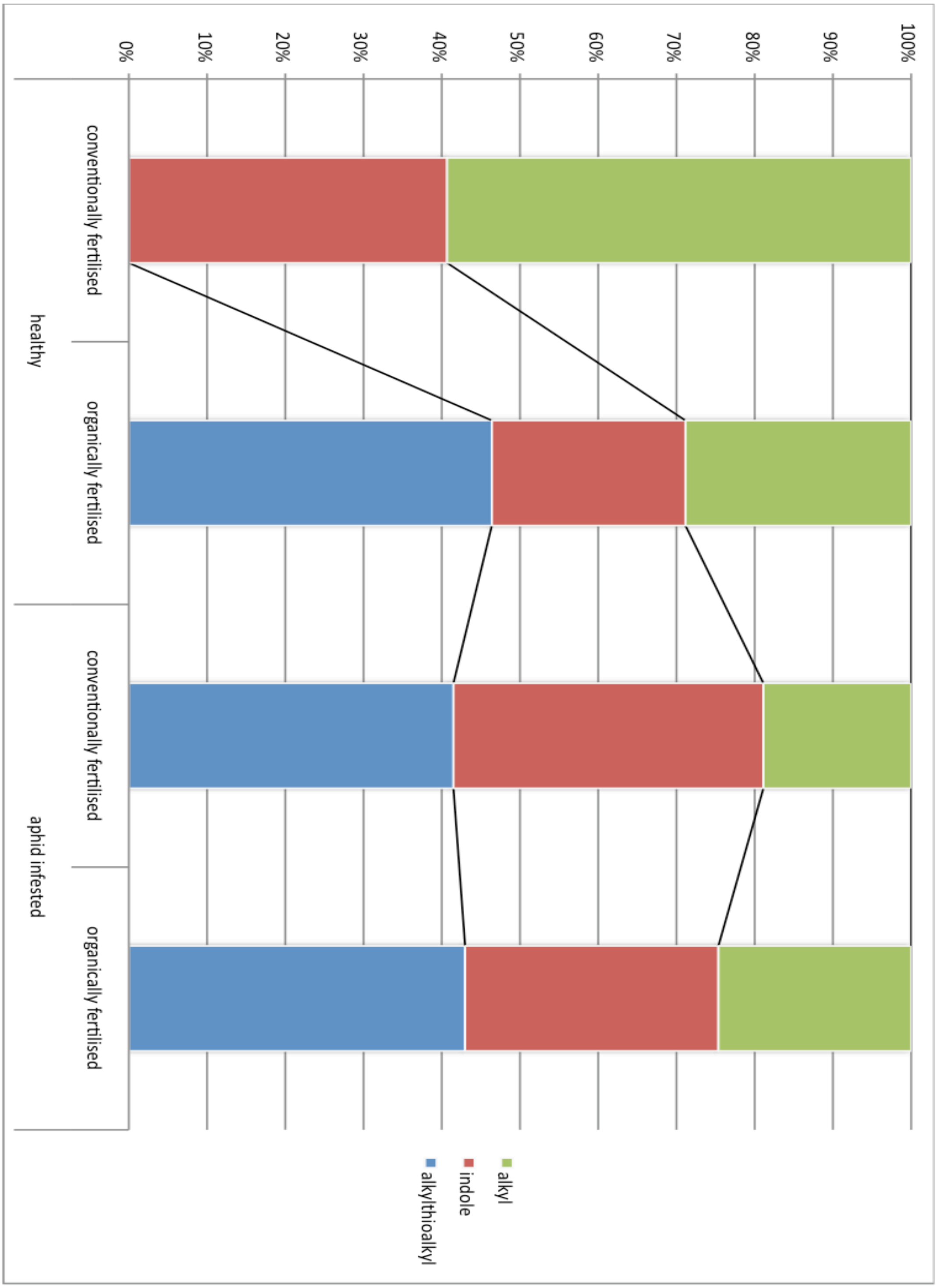

Figure 5.3: Relative levels of glucosinolates present in *Arabidopsis thaliana* when grown under different amendment practices (Plots 2 and 13 from Palace Leas, representing FYM and conventional fertiliser amendment, respectively; conventional amended and FYM amended soils from Nafferton farm) and when under stress from aphid herbivory (*Myzus persicae*).

As a result of herbivory by *Plutella xylostella*, the number of individual volatile chemicals produced decreased. However, the numbers produced were again higher from the conventionally grown plants (10 in total) than the organically grown plants (4 in total). Total volatile levels were also reduced from the infested plants when compared to the non-infested healthy plants. The conventionally grown plants produced an average of 1089ng (±236), and the organically grown plants 306ng (±107) (Figure 5.6).

Infestation with *Myzus persicae* reduced the number of volatile chemicals and the levels at which they were produced, when compared to the non-infested plants. The conventionally grown plants had 10 different chemicals produced at an average level of 1073ng (±102). The organically grown plants had 5 different chemicals produced at an average level of 252ng (±54) (Figures 5.4b, 5.5b).

5. 5. Discussion

The present study was designed to determine the effects of conventional and organic amendment practices on the secondary metabolite-based defences of *Arabidopsis thaliana*. Levels and types of glucosinolates, the major secondary metabolic derived defence molecule in *A. thaliana*, were measured as an example of direct defence. Emitted volatile chemicals were enumerated and the total levels produced measured. Under conventional fertiliser amendment, plants produced higher relative levels of glucosinolates compared to those grown under organic amendment regimes, particularly in respect to increased levels of alkylthioalkyl and indole glucosinolates. Under aphid induced stress relative levels of indole containing glucosinolates was increased regardless of the soil amendment practice.

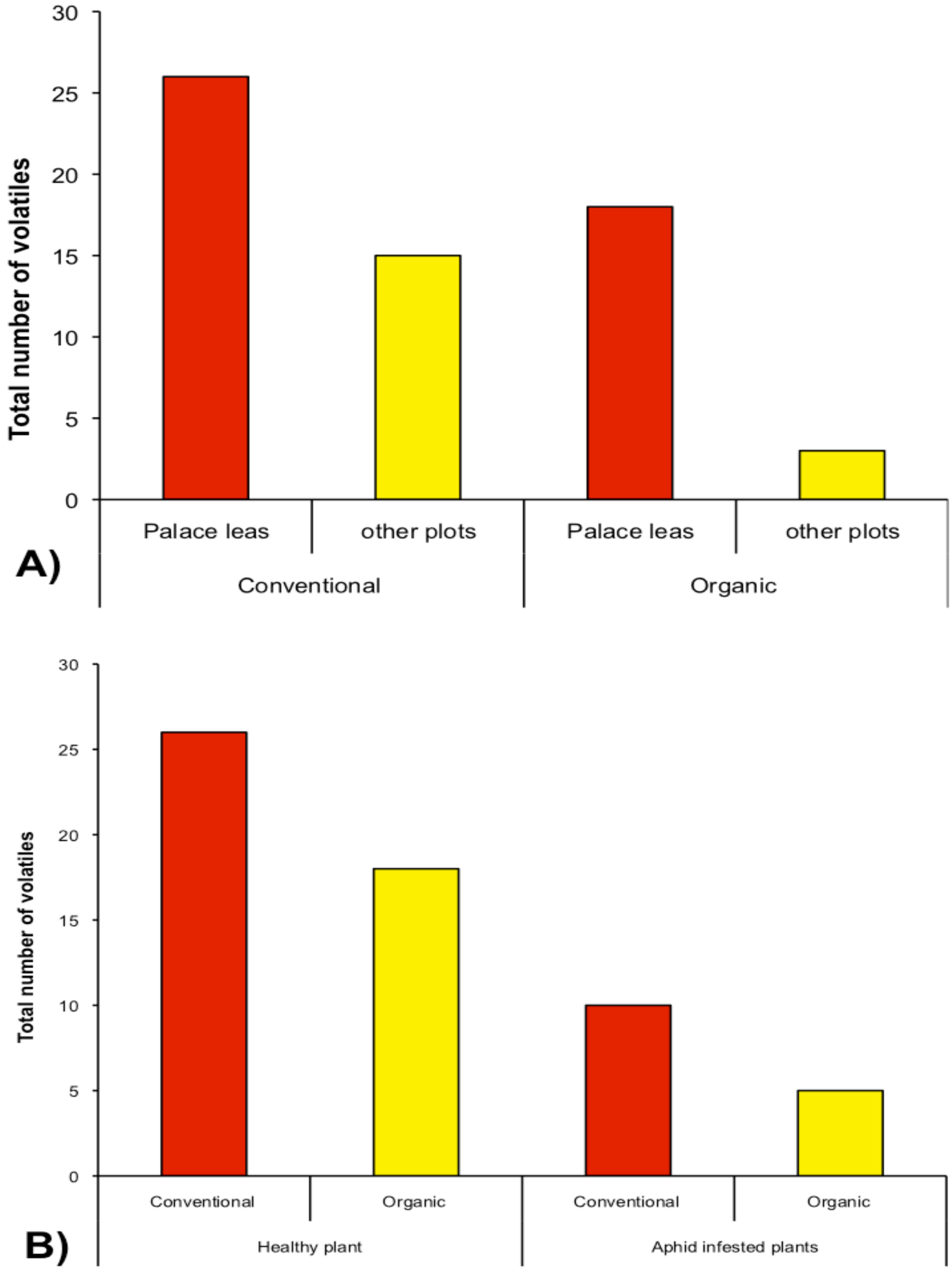

Figure 5.4: Levels of volatile organic compounds released from *Arabidopsis thaliana*. A) total number of different volatile compounds released when grown under different amendment practices. B) total number of different volatile compounds released when grown under different amendment techniques and under conditions of stress from aphid herbivory (*Myzus persicae*). Samples were taken over the period of 24h from the start of infestation

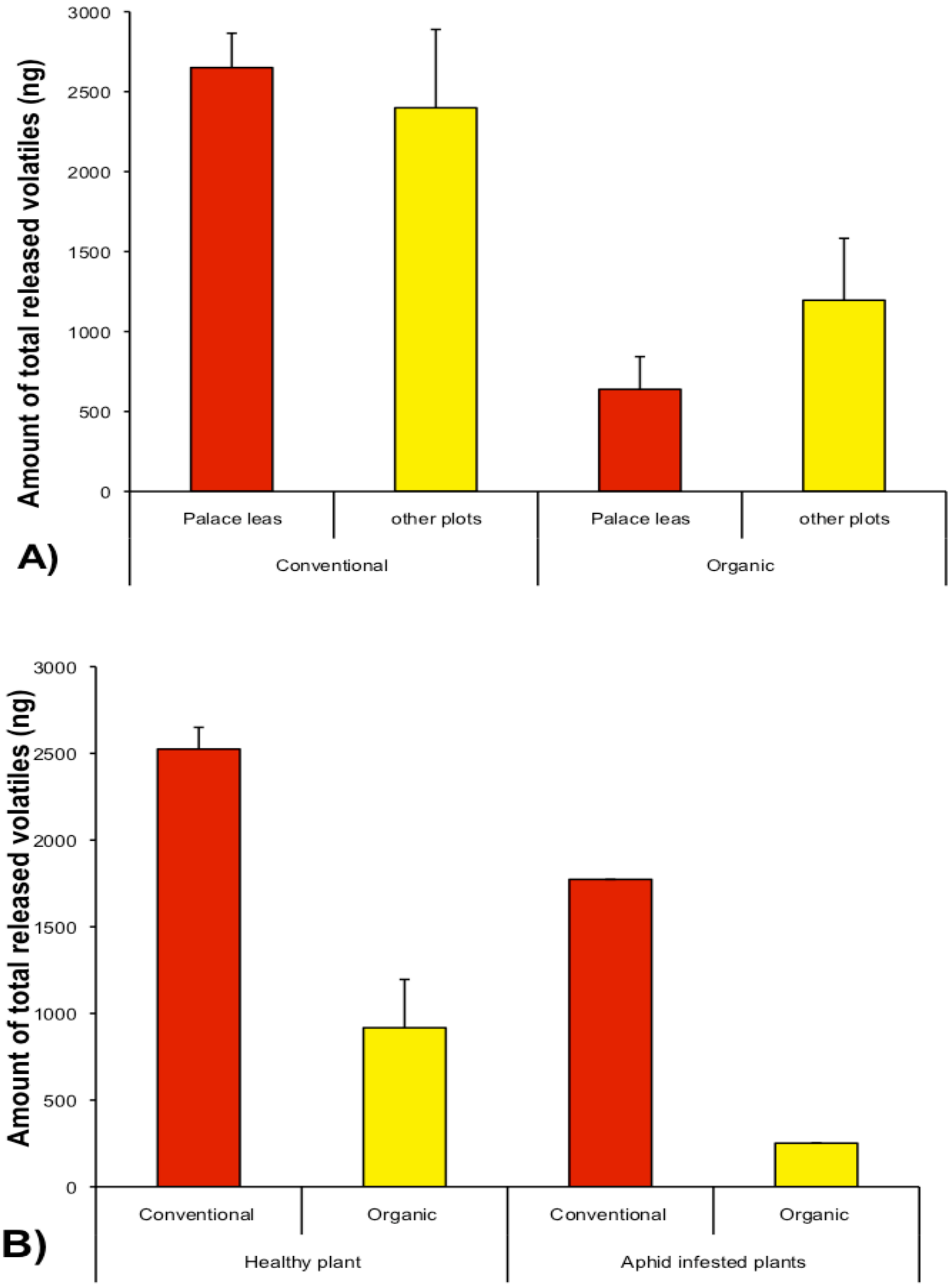

Figure 5.5: Amount of volatile organic compounds released by *Arabidopsis thaliana* over a 24hr period. A) Amount of volatile chemicals released by plants grown in different soil amendment regimes. B) Amount of volatile chemicals released by plants grown in different soil types when under increased stress from aphid (*Myzus persicae*) herbivory. Samples were taken over the period of 24h from the start of infestation.

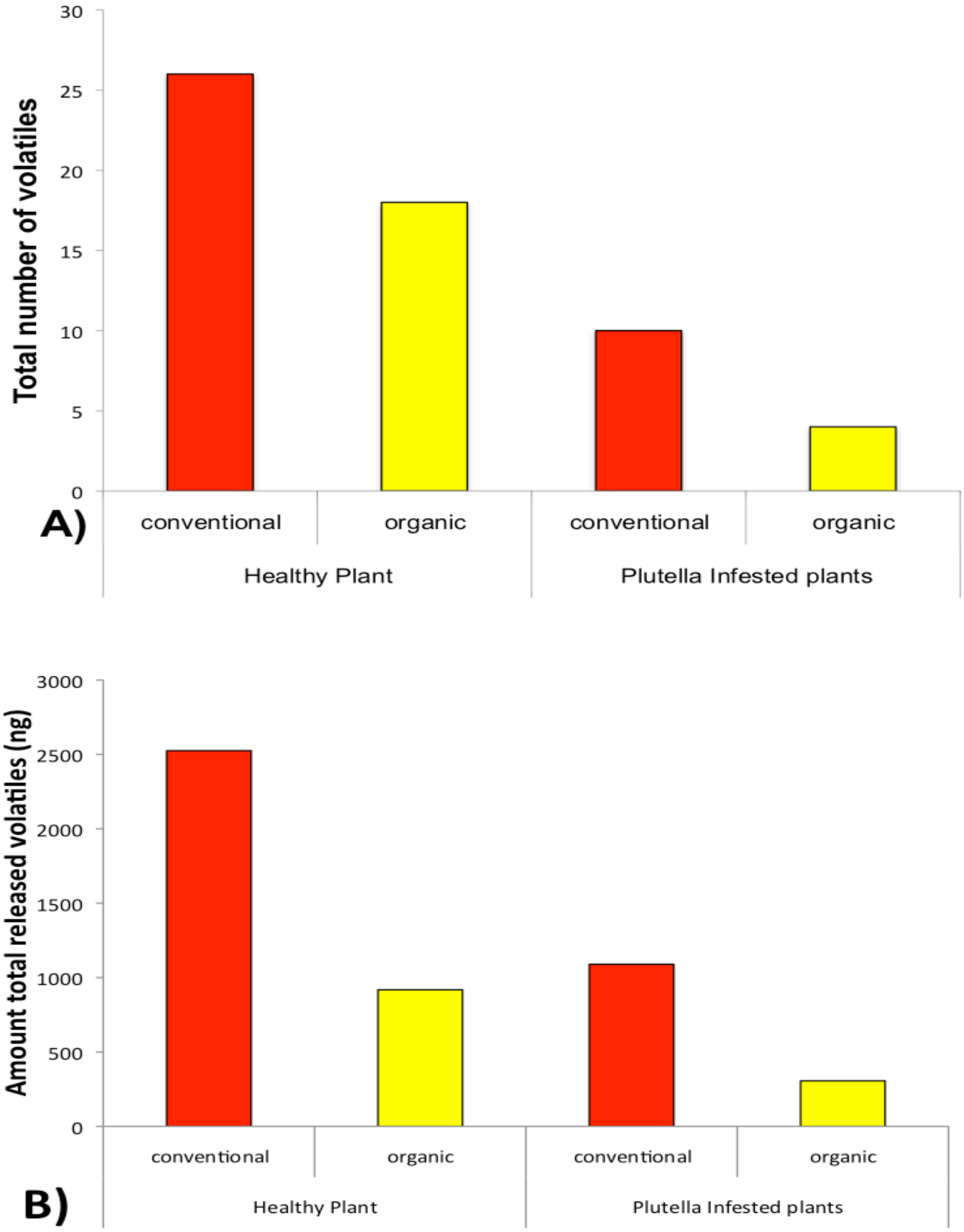

Figure 5.6: Number and levels of volatile organic chemicals released by *Arabidopsis thaliana* when grown in soil with differing amendment practices and when under increased stress from *Plutella xylostella*. A) Number of different volatile molecules released from plants when grown in different soil types and under stress from *Plutella xylostella* herbivory. B) levels of volatiles released from plants in ng when grown on different soil types and when under increased stress from *Plutella xylostella* herbivory. Samples were taken over the period of 24h from the start of infestation.

Volatile production was also affected by fertiliser amendment. A higher number of individual volatile molecules were enumerated from conventionally grown plants and levels were also higher. Under stress from both aphids and lepidopteran larvae, the number and levels of volatiles produced decreased in both treatments when compared to their respective controls. However, conventionally grown plants again showed higher levels.

5. 5. (a) Glucosinolates Present in *Arabidopsis thaliana*

In the present study, soil amendment regimes did not significantly affect the relative levels of glucosinolates in non-infested *A. thaliana* plants. The levels of alkylthioalkyl and indole glucosinolates were similar across all four amendment regimes, long-term organic and conventional amendment and recently converted organic and conventional amendment, while the levels of the alkyl glucosinolates were higher in plants grown in organically amended soils. Previous studies have shown that glucosinolate levels can be altered by the levels of available nutrients, specifically nitrogen and sulphur. Broccoli (*Brassica oleracea* var *italica*) showed a drop in the levels of aliphatic glucosinolates when grown under conditions of decreased sulphur (Schonhof et al., 2007), while broccoli sprouts show a decrease in the levels of glucosinolates under conditions of increased sulphur and nitrogen amendment (Aires et al., 2006).

It was also interesting that the alkyl glucosinolate, gluconapin, showed a decrease in levels in plants grown in organically treated soil. In watercress, gluconapin showed an increase in levels when sulphur amendment was increased (Kopsell et al., 2007). Levels of sulphur appear to directly affect the aliphatic (alkylthioalkyl and alkyl combined) glucosinolates more than the indole glucosinolates, probably as they are produced from a pathway derived from methionine, a sulphur containing amino acid (Zhao et al., 1994). However, it is very likely that the slight decrease seen in the levels of gluconapin is not caused by a lack of sulphur in the FYM amended soil from Palace Leas. Previous studies have shown that, while the conventionally amended plot (Plot 13) had higher available sulphur than the FYM amended plot (Plot 2), biomass sulphur was higher in the organically amended plot (He et al. 1997). These two pools of sulphur when combined provided a significant reserve of sulphur available for plant uptake, indicating that plants grown on these soils should not show any deficiency (He et al., 1997).

Aphid herbivory of *Arabidopsis thaliana* showed some alterations in the relative levels of glucosinolates. There were increases in the levels of indole glucosinolates in plants from both soil types when under stress caused by aphid herbivory, though soil amendment regimes did not cause any significant changes to the relative levels. Aphid herbivory has been shown to cause a decrease by up to 50% in the levels of transcripts of genes involved in the glucosinolate biosynthetic pathways in *A. thaliana* (De Vos et al., 2005). This decrease is also seen in the levels of glucosinolates (Kim and Jander, 2007). However, while there was a decrease in the overall levels of glucosinolates in *A. thaliana,* there was also an increase in the levels of an indole glucosinolate. It is likely that the increase seen was caused by the action of the aphids.

5. 5. (b) Volatile Production in *Arabidopsis thaliana*

The total amount and level of volatiles released by *A. thaliana* were higher in plants grown on conventional soil, irrespective of whether the plants were healthy or under insect attack from either aphids or lepidopteran larvae. Volatile chemicals released from plants are moderated by environmental factors along with biotic stresses. The available supply of nitrogen to the plant can have a direct effect on levels of volatiles produced and released. In maize (*Zea mays*) low nitrogen amendment led to an observed decrease in the levels of volatiles released by the plant when under insect attack (Gouinguene and Turlings, 2002). However, in cotton (*Gossypium hirsutum*) increased levels of nitrogen amendment actually decreased the levels of volatile chemicals produced when under attack from *Spodoptera exigua* (Chen et al., 2008). Further, when under unstressed conditions i.e. the absence of insect herbivory, the conventionally grown plants were found to produce much higher levels of volatiles; there was also an increase in the number of volatile chemical species than in the organically grown plants. There are contradictions in the published studies on how nitrogen can affect plant volatile production, so it is difficult to identify if the nitrogen levels in the Palace Leas plots were affecting the plants. Previous studies of nutrients available to plants in the plots from Palace Leas show that free amino acid levels in the soil were higher in the FYM amended plot, indicating that there are higher nitrogen levels present in this plot (Bol et al., 1998). However, levels of free amino acids are also high in the nitrogen amended plots, so this indicates that there is likely to be very little difference in the available nitrogen between Palace Leas Plots 2 and 13. Indeed, data obtained in Chapter 3 also indicates that these soils do not cause a difference in the plant's utilised nitrogen status. Total chlorophyll levels in *A. thaliana* were not significantly different between plants grown in soil from the two Palace Leas Plots. Chlorophyll levels are often used as an identifier of a crop's nitrogen status (Zhao et al., 2007), so it is assumed that the nitrogen status of the plants grown for the experiment detailed in Chapter 3 is similar between the two treatments.

Under the conditions of the biotic stress, *A. thaliana* showed decreased levels of volatiles being released and also a decrease in the number of species released. There was also a decrease between the soil treatments; plants grown in FYM amended soil showed reduced levels and number of species emitted than in the conventionally grown plants. This was seen when the plants were infested by either a chewing insect (*Plutella xylostella* larvae), or a sap-sucking insect (*Myzus persicae*). However, this result is contrary to other studies. In *A. thaliana*, for example, volatile emissions were higher in both artificially damaged and herbivore damaged plants (Van Poecke et al., 2001).

A difference is often seen in the blend of chemicals released when plants are infested by insects, which have different methods of feeding. In the present study, a decrease in the levels and numbers of volatiles produced between plants infested with either *Plutella xylostella* or *Myzus persicae* was evident though the effects were more pronounced as a consequence of herbivory by *M.persicae*. In parasitoid choice studies in *A. thaliana*, damage by *M.persicae* did not attract the parasitoid wasp, while *P.xylostella* damage did attract the wasp (Van Poecke et al., 2003). In maize (*Zea mays*) there was very little volatile release in response to aphid herbivory, while chewing insects caused large releases of a variety of compounds (Turlings et al., 1998). It is unlikely that *A. thaliana* emits volatile compounds when under aphid herbivory, as the cell structure of the plant is not as damaged as it is with lepidopteran larvae feeding.

5. 5. (c) Conclusions

Overall, amendment regime did not have a pronounced effect on the glucosinolate profile of *A. thaliana*. Any differences seen, such as the decrease in the levels of aliphatic glucosinolates in the plants grown in FYM amended soil, are unlikely to be due to a deficiency in soil nutrients, due to the high levels present in the soil from Palace Leas. The increase in the levels of indole glucosinolates seen in plants from both treatments is due to the plant's response to attack by aphids, as these glucosinolates are known to be metabolised to form toxic compounds in the aphid's gut, unlike aliphatic glucosinolates (Kim and Jander, 2007).

The release of volatile chemicals does appear to be moderated by the amendment practice, as conventionally grown plants exhibit higher levels of volatile emission. However, there is currently not sufficient evidence from the present study to identify whether this is a consequence of soil management practices.

The results from this study indicate that there was little effect of amendment practice on the direct defence, while indirect defence was significantly altered by amendment practice.

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Chapter 6: The use of Structural Equation Modelling to identify Interactions in a Soil-Plant-Aphid Tritrophic System

6. 1. Abstract

The interactions between the soil microbial community and the plant are well known, as are the interactions between the plant and insect herbivores. However, how the soil microbial community can affect insect herbivory and vice versa are less well known. The aim of this study was to identify interactions between fertilizer amendment treatment, soil microbial community, plant and aphid herbivores using observed data and mathematical modelling. The system used in this study was *Arabidopsis thaliana* grown in soil from two plots of the Palace Leas long term field trial. Soil used came from Plot 2, which is amended using farmyard manure, and Plot 13, which is amended using inorganic fertilisers. The observed data sets taken were; Terminal Restriction Fragment Length Polymorphisms for the soil bacterial community, phenotypic data of plant growth and the intrinsic rate of aphid reproduction. Structural Equation Modelling is a powerful tool for estimating the causal relationships between variables. In this study, rate of plant growth, rate of aphid reproduction (r_m) and rate of change of the microbial community were used to identify relationships between these variables. The only significant relationship was a positive correlation between amendment and rate of plant growth, indicating that the use of FYM as an additional amendment increased plant growth. The effect of the rate of plant growth on the rate of aphid reproduction was almost significant and negative, indicating that the rate of plant growth negatively impacted the rate of aphid reproduction.

6. 2. Introduction

Plants are in a system which involves the soil microbiota as well as insect species. There have been many studies on the effect of soil on the plant and on the effect of insect pests on the plant. However relatively few studies have examined the interactions between soil microbial communities, plant and the subsequent suitability of these plants as hosts for herbivorous insects including pest species.

The acquisition of nutrients from the soil is necessary for plant growth.

However, the zone of interaction between soil and plant (the rhizosphere) is not barren; large levels of biomass exist in this zone in the forms of bacteria, fungi and microfauna. These organisms also interact with the plant, either directly or indirectly, for example rhizobacteria are capable of both promoting plant growth and acting as pathogenic agents.

Bacteria that are capable of promoting plant growth are known as Plant Growth Promoting Rhizobacteria (PGPR). PGPR can promote plant growth through a variety of different strategies (Bloemberg and Lugtenberg, 2001). Nitrogen fixing rhizobacteria act as a bio-fertilising agent, as they provide nitrogen in a useable form to plants, either directly through root nodules in leguminous plants, or as free-living rhizobacteria, such as *Azospirillum* species (Steenhoudt and Vanderleyden, 2000). *Azospirillum* are also capable of having a direct effect on the metabolism of plants, as they can produce a variety of phytohormones including gibberellins, cytokinins and especially auxins. Auxin production by this genus is able to alter root morphology in wheat (*Triticum aestivum*); inoculation caused a decrease of root length, while promoting an increase in the number of root hairs (Dobbelaere et al., 1999). A species from this genus, *Azospirillum brasilense*, is also able to promote seed germination in corn (*Zea mays* L.) and soybean (*Glycine max* L.) as is another auxin producing bacterium, *Bradyrhizobium japonicum* (Cassan et al., 2009).

The ability of soil to be suppressive to pathogenic bacteria or fungi is due to the presence of PGPR present in the rhizosphere. The suppression of pathogenic organisms by PGPR occurs by a range of mechanisms, including niche exclusion, nutrient competition, production of anti-microbial metabolites and induced systemic resistance (ISR) or systemic acquired resistance (SAR) within the plant (Sturz and Christie, 2003). The PGPR antagonistic relationship with pathogenic bacteria can lead to the generation of naturally suppressive soils which are often only suppressive to specific pathogens. However, the suppressiveness can be transferred to soils conducive to pathogens and often this causes a broad suppression of disease (Haas and Défago, 2005). The best studied genera of PGPR are *Pseudomonas* and *Bacillus* although both contain pathogenic species along with beneficial species. The actinomycete group of bacteria are also well investigated for their roles (El-Tarabily and Sivasithamparam, 2006). The anti-microbial properties of the actinomycetes, especially *Streptomyces* spp. has been well documented and these species are a target for the production of new medicinal antibiotics (Matve et al., 2001). There is also evidence that green manures and farm yard manures (FYM) are capable of inducing suppressiveness in soils (Zhang et al., 1996). It is likely that some of these effects are caused by an increased competition for nutrients rather than allelopathic effects *per se* (Sturz and Christie, 2003).

While Induced Systemic Resistance and Systemic Acquired Resistance in plants appear to be similar in that they provide the plant with a non-pesticide related defence to pathogens, they differ in their mode of action. ISR is induced primarily by PGPR and is transmitted to the rest of the plant by jasmonate and ethylene signalling, while SAR can be induced by foliar or root elictors and is spread throughout the plant via salicyclate signaling (see Introduction, Figure 1.7). SAR also leads to a build up of pathogenesis related proteins (PR), while ISR does not (Vallad and Goodman, 2004). The jasmonate/ethylene signalling pathway is believed to be associated with necrotrophic pathogens and leaf-chewing insects, while the salicyclate pathway is associated with phloem sucking insects and biotrophic pathogens (Walling, 2000, Rostás et al., 2003). It can be deduced that the activation of a pathway by one type of organism should provide protection against the other and this has been noted experimentally (Moran, 1998). However, there is evidence that the induction of SAR actually provides protection against chewing insects, indicating that the jasmonate signalling pathway is up-regulated along-side the salicyclate pathway (Stout et al., 1999). Chemical elicitation of SAR increased the attractiveness of maize to parasitoids after infestation with lepidopteran larvae compared to plants infested solely with the larvae (Rostás and Turlings, 2008).

PGPR have been shown to provide induced resistance to insect herbivory through the ISR mechanism. For example, Cucumber beetle (*Diabrotica undecimpunctata howardi* Barber) was found to feed significantly less on PGPR treated plants in both glasshouse and field trials (Zehnder et al., 1997a, Zehnder et al., 1997b). Associated with this decrease in feeding was a decrease in the levels of cucurbitacin, a feeding stimulant to cucumber beetle normally present at high levels in cucumber. These studies suggest that ISR has caused a change in the secondary metabolic pathways, shifting production away from cucurbitacin into other, more defensively useful compounds

(Zehnder et al., 1997a).

Some species of PGPR are being sold as commercial adjuncts for agricultural crops, mainly as biocontrol agents (Lucy et al., 2004). There are fewer products available specifically as biofertilising agents, mainly *Azospirillum* spp. which, as noted above, affect plant growth through phytohormone production (Vessey, 2003). However, not all plant species are affected by PGPR, and not all species of PGPR are effective on their own; the use of PGPR commercially is still very much in its infancy.

The interactions between plants and rhizobacteria are not all in one direction, i.e the rhizosphere affecting the plant. Exudate from plant roots contain a large proportion of organic compounds; between 40-90% of carbon transferred to the root is transferred into the rhizosphere (Lynch and Whipps, 1990). This deposition of material into the soil has been shown to have an effect on the rhizobial community and can alter the community structure. Application of artificial exudate to soil at different levels showed large changes in community structure over time (Griffiths et al., 1999). For example maize root exudates applied to the soil showed a decrease in rhizobial diversity combined with an increase in activity of specific bacterial sub-populations (Benizri et al., 2002). Furthermore, mucilage from maize, a significant part of the root exudate, has been shown to increase both microbial carbon, indicating a high rate of micro-organism turnover, and an increase in the number of cultivatable bacteria (Benizri et al., 2007).

Above-ground herbivory, both mammalian and insect, can affect below ground cycling of nutrients through several different mechanisms (Bardgett and Wardle, 2009). Grasshopper herbivory has been identified to increase cycling of nitrogen in grassland and also to affect plant abundance at a medium level of infestation (Belovsky and Slade, 2000). This is likely to be caused both by increased litter and increasing the activity of the fast decompostion cycle. This is an indirect effect on plants, as herbivory does not benefit the plant through mutualistic effects.

There is also an indirect effect of above-ground insect herbivory on mycorrhizal fungi (Gehring and Bennett, 2009), with ectomycorrhizal fungi often being negatively affected by insect herbivory. For example, the presence of an insect herbivore reduced the levels of ectomycorrhizal fungi associated with the roots of susceptible Pinyon Pine trees (*Pinus edulis*), by negatively affecting the mutualism between the tree and fungi (Gehring and Whitham, 1991). However, the response of arbuscular mycorrhizal fungi to insect herbivory is less clear with studies showing both positive and negative responses (Gehring and Bennett, 2009).

Mycorrhizal fungi also have an effect on the insect herbivore, e.g. survival of the moth, *Phlogophora meticulosa* was was shown to be decreased on ryegrass (*Lolium perenne*) associated with arbuscular mycorrhizal fungi (Vicari et al., 2002). It is clear that the effects of the soil microbial community can both directly and indirectly affect the plant through a large array of mechanisms. Studies to date demonstrate that plants are able to directly affect the rhizosphere through root exudates. Insects are indirectly affected by the action of the microbial community via the plant, they in turn can indirectly affect this community.

The aims of this study were to investigate the interactions between soil microflora, the plant and aphid herbivores under selected treatments associated with increasing crop yield using Structural Equation Modelling. Interactions between trophic levels are highly complex and made increasingly difficult to analyse when one or more of the components consists of multiple taxa, such as the soil microflora in this study. In complex systems of this type, responses at one trophic level may act as drivers or products at another level. Modelling system responses under these circumstances is difficult, particularly if quantifying the role of all components in a system response is required.

Structural Equation Models (SEM) and path analysis are statistical techniques for estimating the causal relationships between variables. Path analysis partitions relationships between variables. The response variables identified through this method are driven by predictor variables. The response variables may then carry forward and act as predictor variables for other response variables, therefore forming a network of causal relationships (Elmhagen and Rushton, 2007). The SEM can test the inter-relatedness of each variable within a pathway by examination of their variances. However, two way interactions are often a problem for this method of modelling, in that SEM often cannot identify them properly. SEM are mainly used as a comfirmatory tool rather than an exploratory one; they are better suited to theory testing rather than theory development (Fox, 2002).

Examples of the use of SEM in plant biology to date have predominately focussed on the identification of how plant communities are affected by both abiotic and biotic inputs to a complex system. Plant community structure was identified as positively affecting soil community structure, while soil community structure was negatively affecting plant community structure. There were also significant interactions between plant community structure and soil nitrogen and indirect links between AM fungi and overall functions of the ecosystem (net primary production and evapotranspiration) (Antoninka et al., 2009). Habitat quality has been shown to interact with genetic diversity of *Cirsium dissectum,* as did population size (de Vere et al., 2009)

The soil used in this study was from the Palace Leas long term field trial at Cockle Park Farm, Newcastle University. This trial was set up in 1896 to examine the effects of fertiliser amendment on grassland. Data has been continuously recorded on this site since the beginning of the trial.

6. 3. Materials and methods

6. 3. (1) Outline structure and rationale of materials used

The direct effects of each component in this study are outlined in Figure 6.1. Soil samples from the Palace Leas Experimental field trial were chosen because of the long term amendment regimes used on this site. The two plots used have only been treated with fertiliser, and neither have ever received any pesticide treatment. Whilst Plot 2 has been continuously treated with farmyard manure, Plot 13 has been treated with inorganic chemical fertilisers. This makes them a good model for identifying the effects of different fertiliser amendments on plants.

The plant species used for study was *Arabidopsis thaliana*, the model organism for plant molecular biology. It is fast growing and easily measured for growth experiments.

The aphid species used was *Myzus persicae,* commonly known as the green peach aphid. It is a generalist herbivore of many plant species, including *A. thaliana,* when in its viviparous life stage. This ability to give birth to live young makes it a good model organism for measuring the reproductive rate (r_m), as one female adult has a lifespan of ~20 days and is capable of producing an average of 1.6 nymphs per day (Horsfall, 1924).

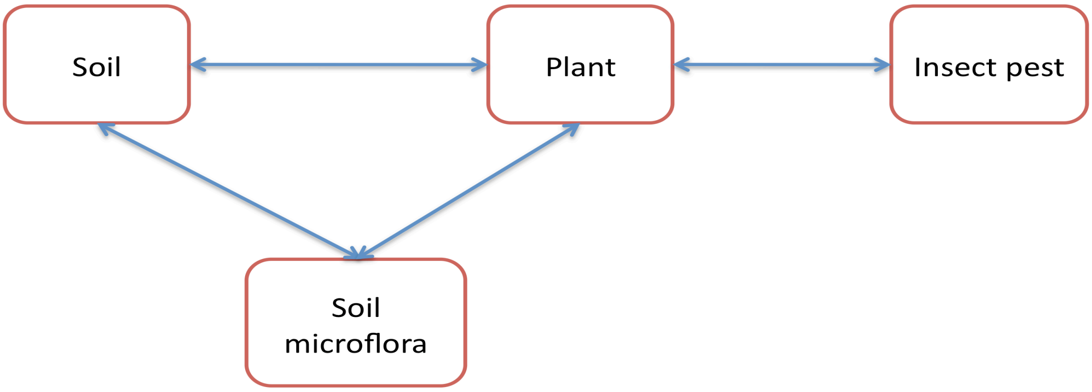

Figure 6.1: Outline of the hypothetical direct interactions present in the system

6. 3. (2) Biological materials

6. 3. (2) (i) Soil collection and amendment

Soil collected from two plots from the Palace Leas long term field trial at Cockle Park Experimental Farm, Newcastle University, which was established in 1896: Soil from Plot 2, which is amended using farmyard manure (FYM) and Plot 13, which is amended with inorganic fertilisers. Plot 2 receives 20t ha^{-1} FYM per annum, while Plot 13 receives 35kg ha^{-1} nitrogen (as ammonium sulphate and sodium nitrate), 60kg ha^{-1} phosphorus (as triple superphosphate) and 67kg ha^{-1} potassium (as potassium oxide; Shiel, Personal Communication). Soil samples were taken at random from across the length of each plot using an auger to a depth of 20cm. All samples were taken two weeks after amendment had been applied. Soil samples were then processed using a 2mm sieve to remove all plant debris and to achieve greater uniformity of the sample. Samples were then stored at 4°C until required.

Soil from Plot 2 was split into two batches before planting. One batch was further amended with FYM from Cockle Park Experimental Farm at a rate similar to the levels of amendment applied to the plot, while the other was left unamended. This provided three treatments of soil: laboratory unamended, organically amended soil (OT); laboratory amended (with FYM), organically amended soil (OA); and conventionally amended soil (CT).

6. 3. (2) (ii) *Arabidopsis thaliana* growth conditions

Arabidopsis thaliana (ecotype C24) seeds were placed on moist filter paper and vernalised at 4ºC for 48 hours. Soil was prepared for potting by mixing with perlite to a final concentration of 30% so as allow adequate drainage. 7.5cm pots were filled and between 2 and 5 seeds were placed onto the surface of the soil. To allow germination, humidity was increased using catering film taped to the pots. After germination, the pots were pricked out to leave one plant per pot.

All plants were grown in a controlled environment room with a 16/8h day/night light cycle and a 22/20ºC day/night temperature cycle. Illumination averaged at 150μmol s^{-1} m^{-2}. Sufficient water was applied throughout the course of the growing period so as to keep the soil moist. A total of 10 plants were used per treatment.

6. 3. (2) (iii) Insect culturing

The green peach aphid (*Myzus persicae*) were cultivated on bean plants for several generations before being transferred to *A.thaliana* for one generation to allow for host-plant switch effects. All aphids were reared at 20ºC with a 16/8h photoperiod.

6. 3. (3) Experimental methods

6. 3. (3) (i) Plant measurements

Measurements of plant growth were taken once the plants had germinated and the cotelydons were fully open. This was considered day zero and all sampling times used this as the starting point. All measurements were taken at the same time of day.

To establish accurate values for these measurements, digital images of each plant were taken every 4 days from day zero. These images were then processed using ImageJ (National Institutes of Health, Washington DC, USA). For each image the workflow consisted of converting from colour to black and white, then processing to form a binary image. Calibration was achieved using a scale provided in each image and the calibration function in the programme. All measurements were then taken using the appropriate function in ImageJ.

6. 3. (3) (ii) Soil sampling

Soil samples were taken from each pot every 4 days to establish any changes in the bacterial community. A total of 1g was taken from each pot and the sample was then frozen until required for DNA extraction.

6. 3. (3) (iii) T-RFLP

6. 3. (3) (iii) (1) DNA extraction

DNA from soil samples were extracted using the method of Griffiths et al., 2000, with a few modifications. All chemicals were obtained from Sigma-Aldrich (Poole, UK) unless otherwise stated. All solutions were made up with DEPC treated water, while all plasticware was DNase and RNase-free. 0.5g (wet weight) of soil was added to a 1.7ml screwcap centrifuge tube containing 0.5g of glass beads. Extraction was performed by adding 0.5ml of CTAB extraction buffer and 0.5ml phenol-chloroform-isoamyl alcohol (25:24:1, pH 8.0). The CTAB extraction buffers consisted of a 10% solution of hexadecyltrimethylammonium bromide in 0.7M sodium chloride mixed in equal parts with 240mM potassium phosphate buffer at pH 8.0. Samples were then lysed using a Hybald ribolyser for 30s at a speed setting of 5.5ms^{-1}. The samples were centrifuged at 13,000g for 5s and the aqueous phase was extracted. Any phenol contamination was removed by mixing with chloroform-isoamyl alcohol (24:1) and centrifugation at 13,000g for 5 minutes. The DNA was precipitated from the aqueous layer using 2 volumes of 30% polyethelene gycol 6000 in 1.6M NaCl. Samples were incubated for 2 hours at room temperature, then centrifuged at 13,000g for 10 minutes. After removal of the supernatant, the samples were washed with ice cold 70% ethanol, followed by air-drying and re-suspension in 200µl Tris-EDTA buffer (10mM Tris, 1mM EDTA).

Due to high levels of humic acid contamination, an extra purification step was required. This was adapted from Cullen and Hirsch, 1998 and Edel-Hermann et al., 2004. Spin columns were made from 0.5ml microcentrifuge tubes which had a small hole placed in the bottom of the tube. A small plug of glass wool was used to support the matrix. 100mg of of polyvinylpolypyrrolidone (PVPP) was added to each column. 2 washes of DEPC-treated water were applied to bed down the PVPP matrix, 300µl of water was added to each tube, left to drip through for 5 minutes, then centrifuged at 1000g for 10 minutes at 10ºC. These columns were prepared in advance and stored at 4ºC. Samples were applied to the columns, allowed to drip through into a collection tube for 5 minutes, then centrifuged at 1000g for 15 minutes at 10ºC. The flowthroughs were precipitated with ethanol/sodium acetate, washed with 70% ethanol and the pellet was resuspended in 50µl of Tris-EDTA buffer. The extraction of DNA from each sample was confirmed using a 0.8% TAE-agarose gel and quantified using a Nanodrop spectrophotometer.

6. 3. (3) (iii) (2) PCR conditions

All samples were amplified using PCR. The primers used were the bacterial 16S rRNA gene primers 8f (AGAGTTTGATPCCTGGCTCAG, Amann et al., 1995) and 926r (CCGTCAATTCCTTTRAGTTT, Muyzer et al., 1995). The 926r primer was 3' labelled with HEX dye. All amplifications were carried out in 30µl reaction mixtures, containing 3µl 10x reaction buffer (100mM Tris-HCl, 500mM KCl, 1% Triton X-100), 1.5mM MgCl, 200µM of each dNTP, 2U of Dynazyme EXT DNA polymerase (Zinnzymes, GRI, UK), 1pmol of each primer and 10ng of DNA. Conditions were 5 minutes at 94ºC then 30 cycles of 30 sec at 94ºC, 30 sec at 56ºC and 30 sec at 72ºC followed by 10min at 72ºC. Samples were checked by running a 5µl sample on a 1.2% agarose gel and a band at ~900 bases was visualised.

6. 3. (3) (iii) (3) Restriction enzyme digest

All samples were digested using *Hha1* restriction enzyme (Promega UK, Southampton, UK). 15µl of PCr product was digested using 6U of *Hha1* for 16h at 37ºC. Samples were de-salted using sodium acetate and ethanol precipitation.

6. 3. (3) (iii) (4) Terminal restriction fragment sizing

All desalted samples were sent to the Durham University Sequencing service to be sized using an Applied Biosystems 3730 DNA sequencer. Results were returned and analysed using the Genotyper software provided by Applied Biosystems.

6. 3. (3) (iv) Aphid reproduction study

Each batch of ten plants was sub-divided into two, each of five plants. One set of plants was left as the control, while the other was infested with aphids. Briefly, two clip cages were attached to the rosette of each plant in the infested pool of each soil treatment. One adult aphid was placed in each clip cage and then left overnight. The following morning the adult female and all but two of the nymphs produced were removed and these nymphs were left to develop to adulthood. Once this point was reached, one aphid was removed. From this point onwards, each morning the number of offspring produced was recorded and all nymphs were removed from the plant. The original aphids were introduced to the plant at day 20, adulthood was reached by day 30 and data were recorded for 14 days until day 44, which was the end of the experiment overall. Using this method it is possible to measure the intrinsic rate of increase (r_m) of an aphid population.

6. 3. (3) (v) Model building

The hypothesis for this model was that soil type and its subsequent treatment would impact on plant growth and then on the aphids themselves. The soil type and amendment would also impact on the soil flora which in turn would impact on plant growth.

To quantify the interactions between these components plant and aphid growth in relation to treatment and soil type were measured along with the response of the soil microbial community present. The length of the major axis of the rosette of the individual plant through time was used as a response variable in non-linear mixed effect models under the assumption that growth would be logistic in the initial phases following the exponential form before reaching an asymptote using the methodology of Pinheiro and Bates (Pinheiro and Bates, 2000). The asymptotic model has three components – a constant, the exponential growth component and the asymptote. We used a self-start model based on initial estimates of asymptote and slope at the mid point of the experiment derived from plots of the observed pattern of growth through time. Mixed effect models were fitted with random effects for slope, and asymptote. The estimated random effects of the exponential growth component of the plants were used as an index of their overall pattern of growth and response to the treatments.

The same analytical approach to model the change in aphid population size was used on each plant, again abstracting the random effects for aphid growth rate for each plant for use in the SEM analysis.

In order to investigate the changes in the soil microflora, a detrended correspondence analysis of the plant was performed by using the T-RFLP segment data set. This effectively provided two axes which summarised the major trends in variation in T-RFLP segment composition across the soil in each plant pot through time. The Detrended Canonical Analysis first axis scores were used as a response variable in a mixed effect model, where the pot from which soil was taken was used as a random effect. Models were fitted with the DCA first axis score as the response and time as the covariate. The random effects for the individual pots then provide an estimate of the rate of changing T-RFLP section composition of the soil in each pot.

These analyses generated a data set comprising: soil type, amendment, plant growth random effects, and aphid population growth random effects and the random effects for the rate of change in soil microfloral composition. Since soil type and amendment were categorical treatments we used polychoric correlation to assess the correlation between treatment, soil type and the other continuous covariates. These correlations were then used as inputs to a path analysis to investigate the relative significance of each component of the pathway. The model was fitted in a Structural Equation Model framework following the methodology of Fox (2002).

The equation used to calculate the model was as follows:

$$\mathbf{y = \text{ø}_1 + (\text{ø}_2 - \text{ø}_1)\, e\hat{}\,(-e^{(\text{ø}3.x)})}$$

ø_1 = asymptote

ø_2 = y intercept

ø_3 = gradient of slope

6. 4. Results

6. 4. (1) Plant growth

The nonlinear mixed effect model of the major axis of the rosette showed a high level of fit with the observed data. The observed data sets of all three treatments followed a pattern of logarithmic growth and this was reflected in the model. Correlation between the fitted model and the observed data was 0.9915 (r^2=0.98, Figure 6.2). Plants grown in the CT soil showed a decreased rate of growth when compared to the organically amended soil (p=0.0049, Table 6.1). There was no difference in rate of growth when OA soil was compared with OT soil.

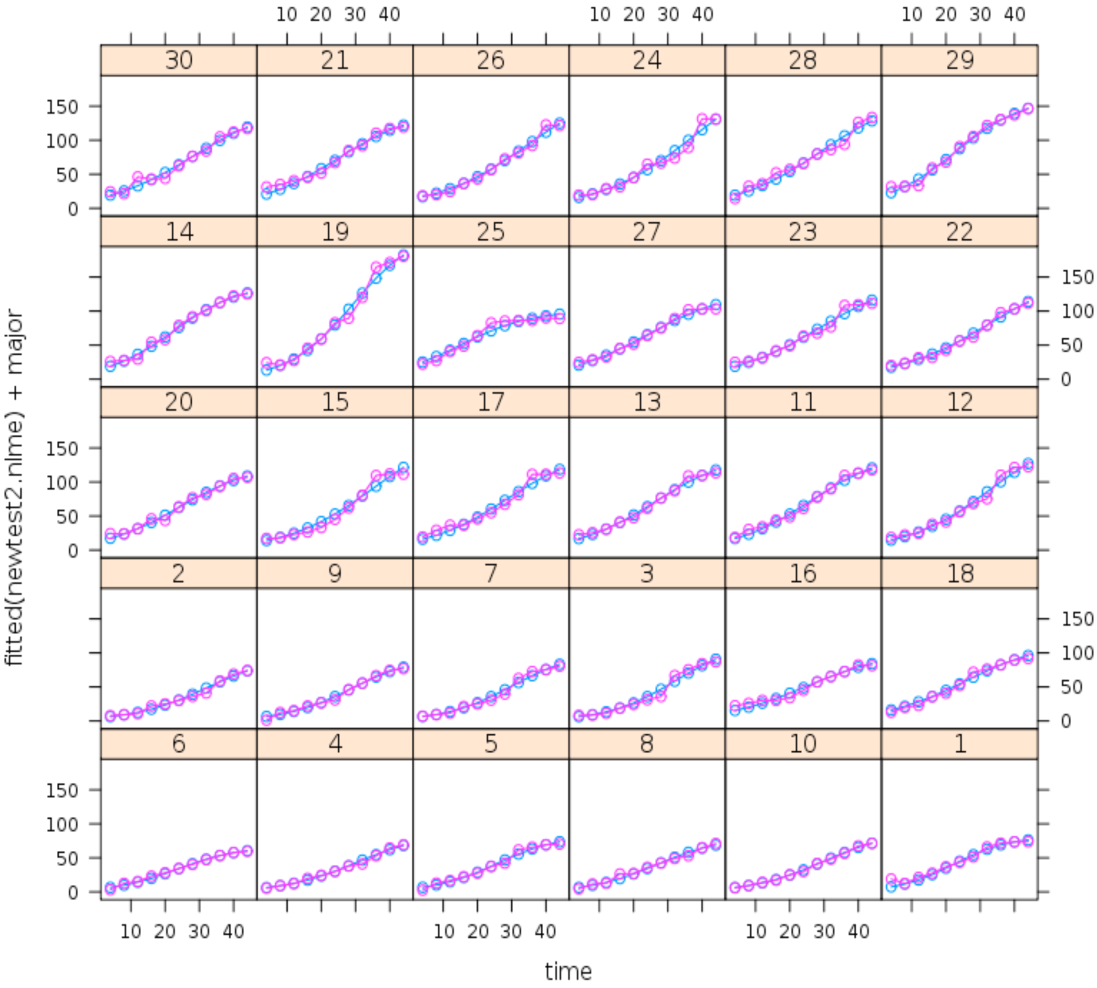

Figure 6.2: Increase of the major axis of the rosette (in mm) over time (in days). Plant ID is indicated above each graph, and treatments linked to these IDs are explained in Table 6.2. Observed data is in pink. Fitted data from the LME model is indicated in blue.

Table 6.1: Table of values from the nonlinear mixed effect model of the major axis of the rosette. Degrees of freedom = 292

	Value	t-value	p-value
intercept asymptote	157.465 ±10.986	14.332	0
conventional asymptote	-67.498 ± 14.857	-4.543	0
organic asymptote	-7.726 ± 15.079	-0.512	0.609
mid point intercept	27.758 ± 1.96	14.136	0
conventional mid point	0.400 ± 2.811	0.142	0.887
organic mid point	-0.276 ± 2.714	-0.101	0.919
intercept growth rate	12.194 ± 0.629	19.389	0
conventional growth rate	-2.659 ± 0.937	-2.836	0.005
organic growth rate	-0.970 ± 0.854	-1.136	0.2566

6. 4. (2) Aphid growth

The linear mixed effect model of the cumulative aphid reproductive rate also shows large correlation with the observed data (correlation was 0.91, r^2=0.97, Figure 6.3). Both model and observed data show logarithmic growth. However, the differences between the CT, OT and OA soils are not significant (Figure 6.4). Interestingly, there appears to be a blocking effect in that two plants in each block of five show higher nymph numbers than the others present within that group and this is reflected in the model. It is likely that this is due to these plants being in an advantageous position of the controlled environment chamber.

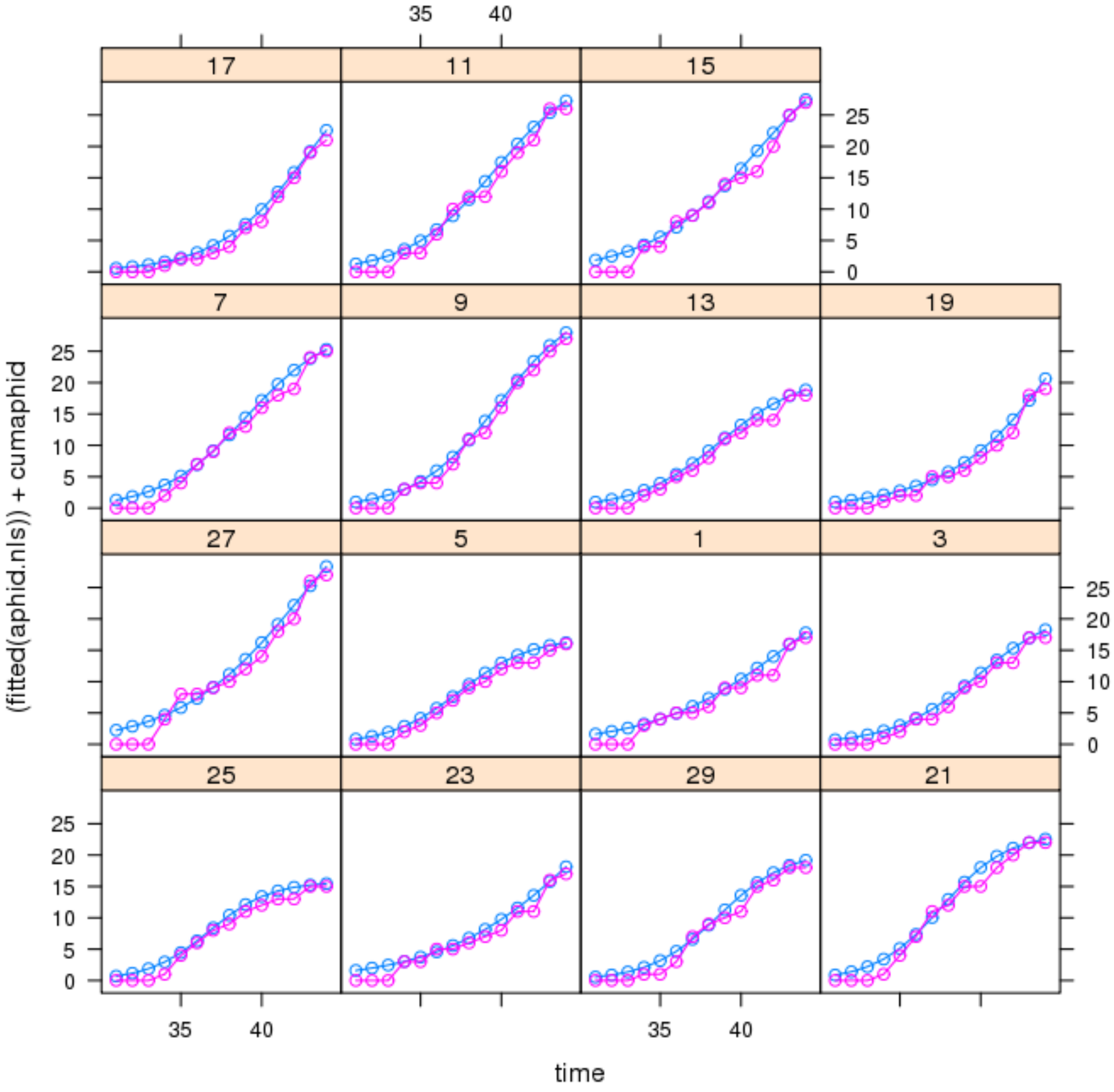

Figure 6.3: Cumulative aphid nymph production over time. Observed data is indicated by the pink data series and the results of the linear mixed effect model is indicated by the blue data series. Plant ID is indicated by the number above each graph.

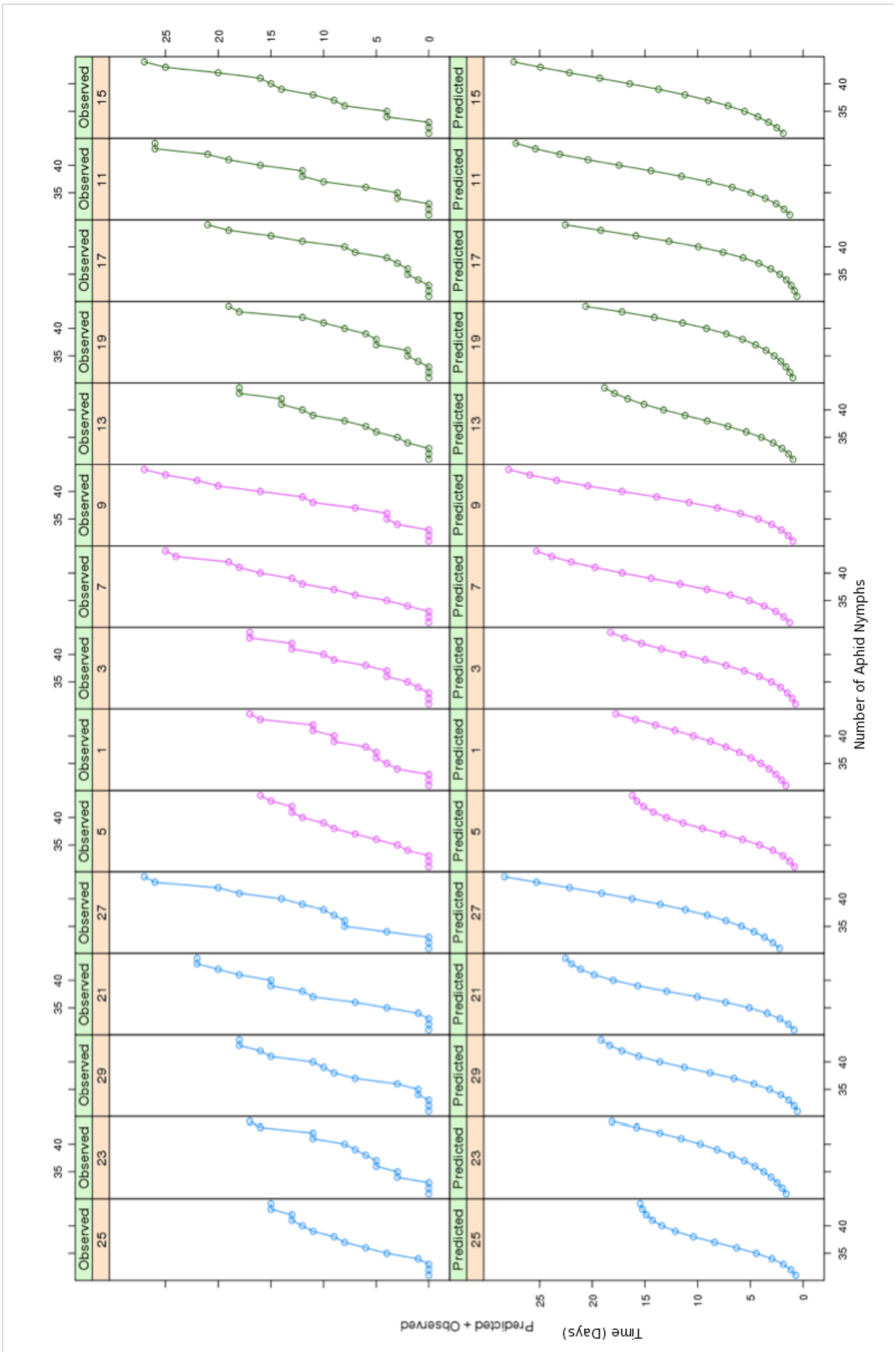

Figure 6.4: Linear effects model (predicted) and observed cumulative aphid reproduction rate. Data in blue are from plants grown in conventionally treated soil, pink are from plants grown in organically treated soil and green are from plants grown in organically amended soil.

6. 4. (3) Changes in T-RFLP

The T-RFLP data were subjected to detrended correspondence analysis. The output summarises the trends in the dynamics of T-RFLP identified taxa in the soil. This was then modelled (Figure 6.5). Trends seen through the linear mixed effect model indicate that there is a mainly positive change in DCA over time in the organically treated and organically amended soils. The conventionally treated soil showed a mainly negative or no change in DCA over time. These trends indicate that the organic and conventional soil treatments were diverging from each other over time.

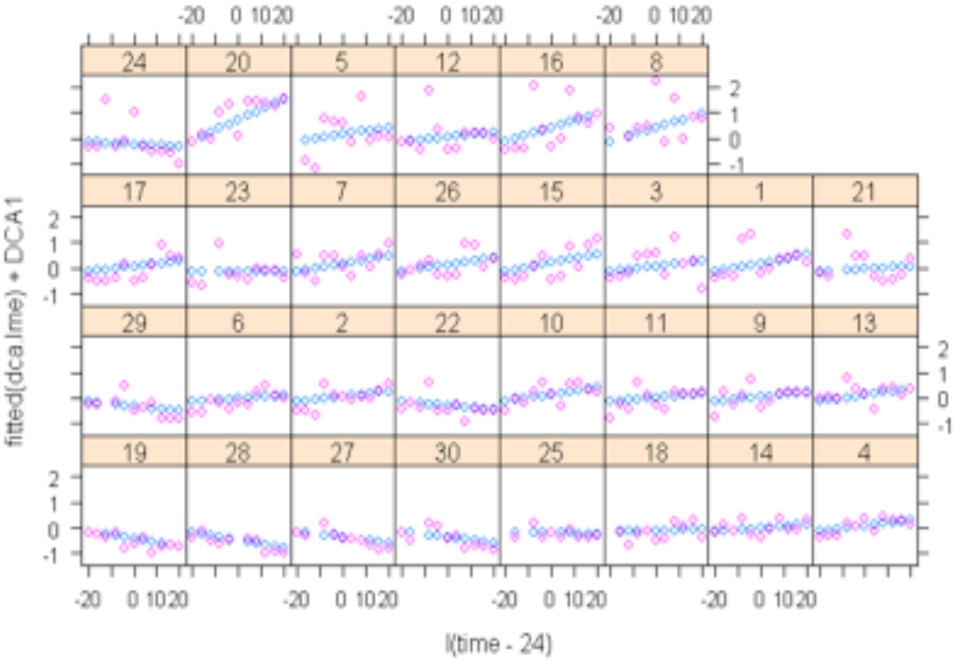

Figure 6.5: Change in Detrended Correspondence Analysis results from T-RFLP data over time. Pink data series are observed data, blue data series are the fitted linear effects model data.

6. 4. (4) Two-step correlations and estimations of bivariate normalcy

Treatment and amendment show a strong positive correlation, as does the rate of growth of the major axis and the asymptote of the major axis. There are medium positive correlations between amendment and the rate of growth of the major axis and the asymptote of the major axis. There are small positive correlations between the DCA of the T-RFLP, amendment and the major axis growth rate (Table 6.2, 6.3). The asymptote value of the major axis shows small negative correlations with treatment, the rate of aphid reproduction and T-RFLP DCA values.

Table 6.2: Table presenting Two-step correlation values (Pearson's correlation) Scal = growth rate

	Treatment	amendment	major axis growth rate	aphid rate of reproduction	TRFLP DCA	Asym major
treatment	1					
amendment	0.5	1				
scal major	-0.03545	0.4045	1			
scal aphid	-0.03264	-0.008804	-0.3224	1		
TRFLP DCA	-0.002884	0.215	0.1913	-0.09197	1	
Asym major	-0.1226	0.4789	0.641	-0.1022	-0.2349	1

Table 6.3: Table of Standard Errors. Scal = growth rate

	Treatment	amendment	major axis growth rate	aphid rate of reproduction	TRFLP DCA
treatment					
amendment	0.1375				
scal major	0.1819	0.153			
scal aphid	0.182	0.1821	0.1637		
TRFLP DCA	0.1821	0.17843	0.1727	0.1807	
Asym major	0.1795	0.1413	0.1054	0.1803	0.1724

Estimates of bivariate normalcy indicate that only three of the interactions show normalcy. The asymptote value of the major axis and scalar value of the major axis show a normal distribution, as do the interactions between T-RFLP DCA values, the asymptote value of the major axis and the major axis growth rate (Table 6.4).

Table 6.4: P-values for tests of bivariate normalcy. Normal values are marked with *. Scal = growth rate

	Treatment	amendment	major axis growth rate	aphid rate of reproduction	TRFLP DCA
treatment					
amendment	$6.987\mathrm{x}10^{-15}$				
scal major	$2.157\mathrm{x}10^{-5}$	$1.423\mathrm{x}10^{-5}$			
scal aphid	$5.837\mathrm{x}10^{-10}$	$4.834\mathrm{x}10^{-10}$	0.01195		
TRFLP DCA	$1.358\mathrm{x}10^{-5}$	$7.566\mathrm{x}10^{-6}$	0.7986*	0.008964	
asym major	$4.17\mathrm{x}10^{-6}$	$5.231\mathrm{x}10^{-6}$	0.8532*	0.002989	0.46*

6. 4. (5) Structural Equation Model output

The relationships between each input of the SEM are shown in Figure 5. The effect of the organic amendment on plant growth was significant (p=0.004), indicating that the addition of organic fertiliser increased the plant's growth. The effect of plant growth on aphid reproduction was almost significant (p=0.067). The estimated value was negative, indicating that the rate of plant growth had a negative effect on the rate of aphid reproduction. All the other interactions were not statistically significant.

These data derived from the model allow the interrelationships between each input in this experiment to be plotted (Figure 6.6). The degree of influence exerted by the laboratory FYM amendment on plant growth is positive and statistically significant (p<0.01).

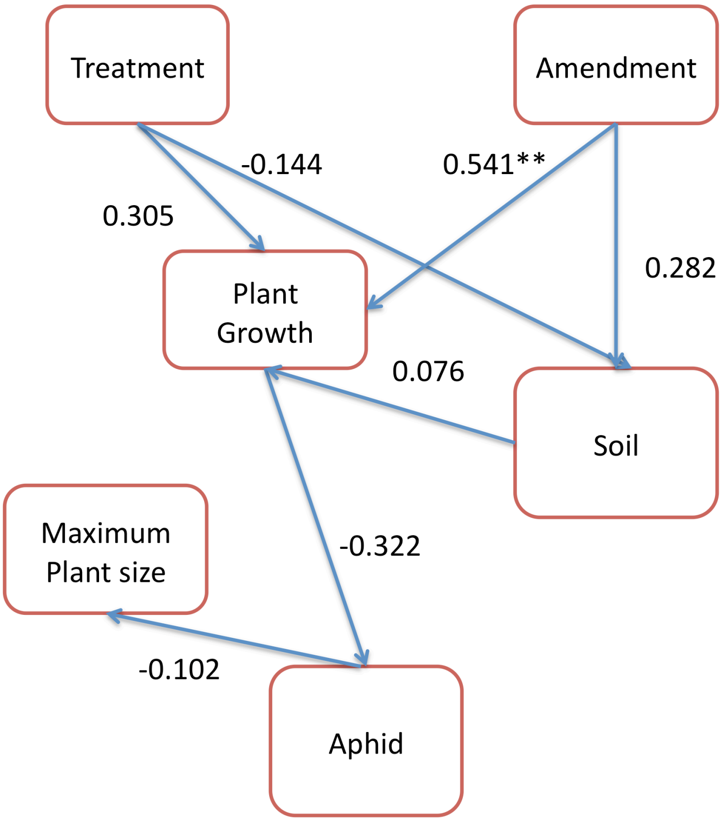

Figure 6.6: Diagram of the interrelationships as described by the Structural Equation Model. Arrows indicate direction of action. Values are the estimate value of the level of influence. ** indicates statistical significance p<0.01

6. 5. Discussion

This study was designed to investigate the interactions in a complex system, that is the interactions between soil microflora, plants and insect herbivores through the use of mathematical modelling techniques. Observed growth data from *Arabidopsis thaliana*, rate of reproduction of *Myzus persicae* and the T-RFLP profile of the soil types used were then modelled using non-linear mixed effect models using the method of Pinheiro and Bates (2000). Further statistical analysis, including correlations and path analysis identified the relationships between each input. The outputs were then fitted into a Structural Equation Model framework. The non-linear mixed effect models indicated that there were significant differences in the rate of growth between OA plants and plants grown in CT soil. No differences were seen in the rate of aphid reproduction dependant on soil type. There was a divergence between the modelled T-RFLP DCA values of the organic soils (both OT and OA) and the conventional soils. The results of the SEM indicated that there was a significant positive interaction between the addition of organic amendment and the rate of plant growth. There was also an almost significant negative interaction between the rate of plant growth and aphid rate of reproduction.

This project was designed as a preliminary study to test the ability of SEM to model the interactions between soil microbial communities, plants and aphids. While the results are not sufficiently conclusive to fully describe this system, the conclusions are still valid. The observed data presented here is a close fit to the non-linear mixed effect model. It also provides evidence that the rate of growth on plants grown in the organic soil and the organically amended soil is higher than that of the conventionally grown plants. This is similar to results seen in Chapter 3 of this thesis, where it is reported that *A. thaliana* grown in the organic soil from Palace Leas grows at a faster rate. Data from the Palace Leas site on hay yields are in agreement with this finding. Coleman et al (1987) reported that average yields from Plot 2 over the course of the experiment (6496t ha^{-1}±2063) are higher than the average yields from Plot 13 (4413t ha^{-1}±1271).

The results presented in Chapters 3 and 4 indicate that the conventional fertiliser amendments used on Plot 13 caused increased stress in plants grown in this soil type. This is supported by mineral levels and ratios presented in Chapter 3 that indicate that the conventionally grown plants are under increased stress. Furthermore, several proteins known to be associated with plant response to stress were shown to be up-regulated in conventionally grown *A.thaliana* (Chapter 4). It is thus likely that the decreased growth reported both in this chapter and Chapter 3 is a consequence of increased stress when plants are grown on conventionally treated soil.

There appears to be no effect of either amendment in the laboratory or amendment on the farm on the rate of aphid reproduction. In Chapter 3 of this thesis, the nitrogen levels of *A.thaliana* grown in these two soil types were similar to one another, based on measurement of the levels of chlorophyll, as a useful indirect method to examine the nitrogen status of a plant (Zhao et al., 2007). Previous studies suggest that the rate of aphid growth tends to be higher on plants that have a higher level of nitrogen present. In both corn and wheat, levels of the aphid pest (*Peregrinus maidis* and *Sitobion avenae* respectively) were higher on plants with increased levels of nitrogen (Wang et al., 2006; Khan and Port, 2008). Due to these observations, it is possible to infer that because the rate of aphid reproduction were similar between the treatments and amendment the nitrogen status of the plants was also similar.

A blocking effect was also observed in the present study, with higher rates of aphid reproduction present in two plants from each treatment. It is likely that this will have been caused by these plants being placed in an advantageous place within the controlled environment chamber.

The data presented in this study indicate that the microbial communities present in the organic (OT) and organically amended (OA) soils are diverging away from those present in the conventional (CT) soil type. There is often a change in the microbial community seen on the addition of organic fertiliser, due to the addition of new species from the fertiliser (Edel-Hermann et al., 2004, Perez-Piqueres et al., 2006). Organically amended soils can have distinctly different microbial community structures compared to those of conventionally amended soils, due to the long-term addition of organic fertilisers (Widmer et al., 2006). Community level physiological profiling of plots from Palace Leas show that there is a distinct separation between the two plots used in the study presented here i.e. in Plots 2 and 13. Phospholipid-derived fatty acid analysis also indicates separation between the organically amended and conventionally amended plots (O'Donnell et al., 2001).

The plant itself may also be affecting the microbial community through rhizodeposition (Paterson et al., 2007). Root exudates are capable of increasing the metabolic activity of specific sub-populations of rhizobacteria, although this appeared to be associated with a decrease in the diversity of the rhizosphere (Benizri et al., 2002). However, there is evidence that the effect of plant species is not a significant driver in the structure of microbial community;

Kennedy et al. (2004) found that the addition of nitrogen to grassland soil affected the microbial community, while the plant species did not have a great effect. In the present study it is difficult to identify exactly what is causing the observed divergence of the microbial communities but it is likely to be due to a number of factors. It is also difficult to identify which taxa are changing in abundance.

The SEM identified a significant positive effect of organic amendment. This effect indicated that the rate of plant growth was increased by the addition of organic fertiliser. It is likely that this is caused by the addition of additional nutrients into the soil from FYM. While the soils by themselves are able to provide nutrients needed for plant growth, the addition of an amendment will often increase growth and therefore yield. The Mineral Balance hypothesis, also known as Trophobiosis, states that the optimal balance of nutrients taken up by plants will ensure maximum plant growth and increased non-gene mediated resistance (Chaboussou, 1985, Phelan et al., 1996, Phelan, 1997). Organic soils appear to be capable of buffering the uptake of minerals to plants (Phelan, 1997). It is possible that the levels of nutrients present in the organically amended and conventionally amended soils used in this study are at 'optimal' levels for the growth of *A.thaliana*.

There is an almost significant negative effect on the rate of aphid reproduction, caused by the rate of plant growth. This indicates that with an increased rate of plant growth, there is a decrease in the rate of aphid reproduction. As rate of plant growth is affected by the presence of organic amendment, it is possible that the decrease in aphid reproductive rate is caused by either Induced Systemic Resistance or Systemic Aquired Resistance. Both ISR and SAR can be induced in plants through the addition of organic amendment (Vallad and Goodman, 2004). The action of aphid feeding is known to activate salicylate mediated pathways, which are responsible for pathogen resistance in plants (Gatehouse, 2002). SAR is also implicated in the release of salicylate as a signalling molecule and is often considered a more generalised plant response than ISR, as it can be caused by a wider range of factors. No ISR-inducing bacteria were identified as being present in the soil, it is more likely that SAR is being induced in these plants, thereby decreasing aphid reproductive rates. However, there is a possibility that this decrease in aphid reproductive rate is actually mediated through non-genetic means and is a consequence of mineral balance, as predicted by the Mineral Balance Hypothesis. For example, there is evidence from Chapter 3 of this study that levels of boron are lower in plants grown in the conventional soil in this study. Beanland et al. (2003) indicated that decreased levels of boron led to increased developmental performance of three insect herbivores on soybean (*Glycine max*). It is possible that a similar effect is being seen here. Furthermore, the increased plant growth rate means that the rate of metabolism is higher in these plants. They are, therefore, using nutrients at a higher level, which would prevent them from being removed from the phloem by aphids (Chaboussou, 1985).

While these two interactions are statistically significant or almost statistically signficant (plant growth rate and aphid performance, respectively), the remaining interactions show no statistical significance. However, this does not mean that these interactions do not occur within the system at both a model level or at an experimental level. As noted above, this is an initial study designed to identify these interactions, and also identify areas for further study. Additional observed data, including some on the effects of different soil amendments would help strengthen the model. The majority of bivariate interactions in this study were not found to be normal and it is likely that this is the major cause of a lack of statistically significant interactions. While this model in this study has not identified many distinct above- and below-ground interactions, there is evidence from published studies that SEM can be used to identify these interactions. Grassland plant community structure was shown to be negatively affected by soil community structure, while the plant community structure positively affected microbial community structure. There appeared to be a significant degree of interconnectedness between the below- and above-ground communities (Antoninka et al., 2009)

The T-RFLP method is a useful tool for the identification of changes in the microbial community over time. However, due to the limited number of fragments possible within the amplicon, a large number of taxa will share the same fragment length, which makes identifying taxa through this measure difficult at best. Other methods, such as Denaturing Gradient Gel Electrophoresis (DGGE), which allows identification of individual bands through DNA sequencing would be more suitable for this model (de Souza et al., 2004).

Levels of rhizobacteria that directly affect the plant could then be quantified and inserted individually into the model. Tracking each taxon through time would identify how much it affects plant growth. Unfortunately in the present study this has not been possible. The addition of soil and plant mineral levels over time to the model would also help identify the effect they have on plant growth and aphid reproductive rate.

While SEM is an effective modelling technique for the identification of interactions between variables, it cannot provide the definitive mechanisms of these relationships (de Vere et al., 2009). This modelling system also has other significant limitations. Data requirements are significant in that there needs to be a high level of replication along with a large range of measured variables. Coupled with this, the SEM strategy cannot give results that extend beyond the data provided to the model, as it is a comfirmatory method. There is no ability to prove causation, although causal hypotheses can be falsified (Grace and Pugesek, 1998). Despite these limitations, SEM remains a powerful multivariate statistical tool.

Overall, the use of Structural Equation Modelling in the identification of interactions between inputs to a soil-plant-insect herbivore tritrophic system has been examined. The results indicate that this type of model is suitable for this role, albeit additional observed data are required to increase the statistical significance of the interactions.

6. 6. References

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Chapter 7. General Conclusions

The aims of this thesis were to examine the effects of organic and conventional amendment practices on the soil microflora, and the subsequent effects on plants grown using these regimes. The studies outlined in this thesis used *Arabidopsis thaliana* as the model plant organism as a completed annotated genome sequence is available. Soil was taken from the Palace Leas hay plots long-term field trial at Cockle Park Farm, Newcastle University, which was established in 1896 as a trial to identify the best amendment methods to regenerate old grassland. Two plots were used from this trial: Plot 13, which receives inorganic (mineral) amendment and Plot 2, which receives organic farmyard manure (FYM) (Shiel, Personal Communication).

The effects of the original amendment practices and the addition of further amendment on the soil bacterial and fungal community structure (Chapter 2), and the subsequent impact of these soil treatments on growth of *A. thaliana* (Chapter 3) were investigated. Phenotypic data, including rosette area, diameter, average leaf number and dry weights of the rosette, inflorescence and seed yield was monitored. Levels of chlorophyll, available tissue nitrate and several inorganic minerals were also measured. Differential expression of the proteome between *A. thaliana* grown on organic or conventionally amended soil were studied using 2D-Polyacrylamide Gel Electrophoresis and image matching software and differentially expressed proteins identified using Matrix Assisted Laser Desorption Ionisation Time of Flight Mass Spectrometry (MALDI-TOF MS) (Chapter 4). The effects of soil amendment practice on both direct and indirect chemical defences in the model plant in response to insect herbivory were also investigated, using two species of insect pest with very different modes of feeding (Chapter 5). Relative glucosinolate levels were measured using High Pressure Liquid Chromatography coupled with a mass spectrometer when *A. thaliana* was under as a consequence of herbivory stress from the green peach aphid (*Myzus persicae*). Levels of volatile chemicals release in the absence of pest pressure, and in response to herbivory from *Plutella xylostella* larvae (Lepidoptera) as a chewing insect and herbivory from *Myzus persicae, as a sap-sucking insect* were measured using Gas chromatography. Finally, phenotypic data obtained from plant growth, soil bacterial community structure data and aphid rate of reproduction data were used to construct a Structural Equation Model (SEM) to identify some of the inter-relationships between each variable input (Chapter 6).

The microbial community present in the two soil types used here were analysed using Terminal-Restriction Fragment Length Polymorphism (T-RFLP). Soil from each of the two plots used in this experiment were either amended with FYM, mineral fertiliser or left unamended, thus there were 3 treatments per plot. Microbial DNA was extracted from soil samples over a period of 7 weeks so that changes in community structure could be measured over time. Detrended Canonical Analysis (DCA) was used as a statistical test to identify how the soil type, addition of amendment or time affected the microbial community structure. The results showed that there was no significant effect of the original treatment on the bacterial or fungal community (p=0.263, p=0.342). However, the addition of laboratory amendment showed a significant effect (p=0.047). There was a significant difference between laboratory amendment with inorganic fertilisers and FYM (p=0.0467). Time, as a covariate, showed no significant effects on either the fungal or bacterial communities. It can be concluded that the addition of amendment has a greater effect on the microbial community structure in the soils from the Palace Leas field trial than the original treatments.

The phenotypic measurements taken in Chapter 3 identified that throughout the course of this study, *A. thaliana* grew at a faster rate when grown in the FYM amended soil than when grown in the conventionally amended soil. However, this increased rate of growth was only observed in the initial and middle phases of growth; by the end of the time period, plants on both soil types were of similar size. Dry weights, which can be considered as a measure of yield of *A. thaliana,* were only statistically significantly higher in plants grown on FYM amended soil in the weight of the rosette, while inflorescence and seed yield were both similar between the two plots. Reported average hay yield levels from the Palace Leas Field trial indicate that the organic plot has cropped higher levels than the conventionally amended plot over the course of that trial (Coleman et al., 1987). The findings from the present study are in contrast to most other comparative yield studies which indicate that organic amendment yields are lower than best practice conventional agriculture (Leake, 1999, Aventis Crop Science, 2000). However, some long term field trials, such as the

Broadbalk and Hoosfield trials at Rothamsted Research Station show comparable yields between organic and conventional amendment (Rothamsted Research, 2006). Other agricultural systems that encourage the use of high levels of organic amendment, such as the System of Rice Intensification, show significantly higher yields than conventional practices (Uphoff, 1999).

Other parameters studied included levels of minerals, available tissue nitrate and chlorophyll levels (Chapter 3). Chlorophyll levels were not significantly different between treatments. As chlorophyll is a reliable indicator of utilised nitrogen status of plants (Zhao et al., 2007) it can be concluded that the nitrogen status of the plants is similar. Boron, calcium, magnesium and potassium are all essential minerals for plant growth and there is evidence that the K/Ca and K/Mg ratios can be used as indicators of increased plant stress (Chaboussou, 1985). Boron is an important micronutrient in its role as a co-enzyme. Results from the present study indicated that boron levels were lower in conventional plants, which may indicate that these plants are deficient in this mineral, which in turn would affect growth. Farmyard manure can increase levels of boron available to plants (Sharma et al., 1999), which may well explain the increased uptake. Levels of the other three minerals examined in this study were also higher in plants grown on FYM amended soil and may reflect increased bio-availability of nitrogen. Increased availability of nitrogen from the soil often leads to increased uptake of inorganic cations and it is likely that this is the case here (Kirkby and Knight, 1977). Mineral ratios have been indicated as predictors of plant health; fruit trees show increased resistance to scale rot when the K/Ca ratio is in favour of K (Chaboussou, 1985). In wheat, drought tolerance is increased with a high K/Ca ratio (Majid et al., 2007). A higher K/Ca ratio may also indicate high levels of protein synthesis (Steward et al., 1962). In the study reported here, the K/Ca and K/Mg ratios were both lower in conventionally grown plants, indicating again that these plants were under increased stress.

Overall, it appears that *A. thaliana* grew at a faster rate in FYM amended soil compared to those grown in mineral amended soil, possibly due to increased stress reducing the rate of growth in the plants grown on conventionally amended soil. However, there was no convincing evidence that higher levels of biomass, or increased yields, were produced by organic management practices.

The influence of the type of soil amendment used on protein expression profiles was also a focus of the study as it provides information as to how the plant is able to respond to differing growing conditions. A total of 46 protein spots were shown to be differentially regulated in response to fertilizer regime, and seven of these proteins were identified using MALDI-TOF MS analysis. A further two proteins were identified; however theoretical and observed pI values did not match. From these identified proteins, several are known to be involved in the mediation of plant stress response, while the others are involved in hormone metabolism, photosynthesis and cell signalling.

Proteins up-regulated in plants grown on conventionally amended soil and known to be involved in the plant stress response were glutathione-S-transferases F3 and F4 and Heat Shock Cognate Protein 71 (HSP71). HSP71 is generically up-regulated in response to stress, due to its role in folding new and damaged proteins and are often used as a marker to indicate increased stress (Timperio et al., 2008). Glutathione-S-transferases play a role in the detoxification of stress induced metabolites, such as lipid peroxidases, which are formed as a consequence of the oxidative burst mechanism (Ferry and Gatehouse, 2009). However, two other proteins, both lipoxygenases, which are usually up-regulated as part of the plant's stress response were actually down-regulated in *A. thaliana* grown on conventionally amended soil. Lipoxygenases are involved in the production of Reactive Oxygen Species (ROS), part of the oxidative burst mechanism when a plant is under either abiotic or biotic stress (Bhattacharjee, 2005). The other proteins identified as being differentially regulated in this study were nitrilase 1, oxygen-evolving enhancer protein 1, Annexin D6 and 14-3-3 general regulation factor 14. Nitrilase 1 converts a precursor chemical into Indole-3-acetic acid (IAA), one of the major plant hormones. Higher levels of this protein are seen in the conventionally grown plants, indicating that levels of IAA are likely to be higher in these plants. Under salt stress, *A. thaliana* has been shown to exhibit increased levels of mRNA transcript for this protein, indicating a possible role for IAA in stress (Bao and Li, 2002). It is possible that in the present study a similar process is occurring and that this up-regulation is a consequence of the plant being under to stress. Annexin D6 is part of the annexin family in *A. thaliana* and is a ubiquitous Ca^{2+} binding protein involved, amongst other roles, in the regulation of ROS (Mortimer et al., 2008). The mRNA transcripts of a closely related *A. thaliana* annexin have been shown to be up-regulated under conditions of salt stress (Lee et al., 2004). 14-3-3 general regulation factor 14 is part of the 14-3-3 protein family which has a role in intra-cellular signalling pathways (Wu et al., 1997). In the present study, this protein was shown to be up-regulated when grown in conventionally amended soil. Other studies have shown proteins in this family to be up-regulated when the plant was under conditions of salt stress and mineral deficiency (Xu and Shi, 2006), while further research has shown an association with the formation of ROS (Elmayan et al., 2007). In the only comparable study to date, Lehesranta et al (Lehesranta et al., 2007) identified a large number of proteins which were up-regulated in organically grown potatoes that had roles in either stress response mediation or direct defence of insect herbivory. These findings are in contrast to our study, where conventional amendment practices were shown to cause increased stress.

There have been many unsubstantiated claims that organic farming practices result in crops being inherently more resistant to biotic stress, including insect pests. The effects of amendment practice on the direct and indirect secondary metabolism based defences against insect herbivory in *A. thaliana* were therefore investigated (Chapter 5). Glucosinolates, the major direct defence molecule produced in *A. thaliana,* were identified by Liquid Chromatography-Mass Spectrometry and their relative levels in plants were measured both when under no biotic stress and in response to aphid induced stress (Chapter 5). Amendment regime did not significantly alter the levels of glucosinolates present in *A. thaliana*. Relative levels of the two major groups identified, the indoyl- and alkylthioakyl-glucosinolates showed similarities between both soil amendment treatments, while there was a small increase in the levels of alkyl-glucosinolates in plants grown on soil amended with FYM. Under aphid herbivory, there was an increase in the levels of the indoyl-glucosinolates in the infested plants. The size of this increase was similar between the two soil types, indicating that it was caused by infestation and not amendment regime. *A. thaliana* has been shown to increase levels of these glucosinolates when under aphid herbivory, as these compounds are broken down into toxic molecules in the aphid gut, unlike other forms of glucosinolates (Kim and Jander, 2007).

The levels of volatile chemicals released by the plants when under conditions of no biotic stress, or when infested by either lepidopteran larvae or aphids (i.e. two species with distinct feeding behaviours) were measured to identify the changes in indirect defence caused by amendment treatment. Volatile emissions were higher in conventionally grown plants, regardless of amendment treatment or type of insect herbivory. When under unstressed conditions, i.e. the absence of insect infestation, the conventionally grown plants produced much higher levels of volatiles and there were an increased number of volatile chemical species present than in the organically grown plants. Under the conditions of biotic stress used in this study (insect herbivory from a lepidopteran larvae, *Plutella xylostella* and from an aphid *Myzus persicae*), *A. thaliana* showed decreased levels of volatiles being released and also a decrease in the number of species released. There is also a decrease between the soil treatments; organically grown plants show reduced levels and number of species emitted compared to the conventionally grown plants. This is seen irrespective of whether the plants are infested by chewing insects (*Plutella xylostella* larvae) or sap-sucking insects (*Myzus persicae*). However, this result is contrary to other studies. In *A. thaliana* volatile emissions were higher in both artificially damaged and herbivore damaged plants, (Van Poecke et al., 2001).

Mathematical models were used in an attempt to identify the interactions in this complex system (Chapter 6). Observed phenotypic data from the life span of *A. thaliana*, the rate of reproduction of *Myzus persicae* and microbial community structure of the soil types used were modelled using non-linear mixed effect models (Pinheiro and Bates, 2000). Path analysis and Structural Equation Modelling (SEM) were then used to identify and evaluate the inter-relationships between variables. The non-linear mixed effect models indicated that there were significant differences in the rate of growth between plants grown on FYM amended soils and plants grown in conventionally amended soil. However, no differences were seen in the rate of aphid reproduction dependant on soil type. There was a divergence between the modelled T-RFLP DCA values of the organic soils and the conventional soils. The results of the SEM indicated that there was a significant positive interaction between the addition of FYM amendments and the rate of plant growth, indicating that the addition of FYM amendment increased plant growth rate. There was also an almost significant negative interaction between the rate of plant growth and rate of aphid reproduction, indicating that at an increased rate of plant growth, the rate of aphid reproduction was reduced. This study should be viewed as preliminary, as only the two interactions detailed above showed statistical significance. Many of the bivariate interactions did not show normality, which is likely to have prevented the SEM from correctly evaluating these interactions. However, there is evidence from both this study and from published studies that SEM can be used as a valid technique to identify above- and below-ground interactions in plant systems (Antoninka et al., 2009).

Agriculture in the UK has been moving towards minimal environmental impact while maintaining the high yields allowed by ‘Green Revolution’ practices. This has been driven by increased legislation from the Government to prevent environmental damage, for example the banning of persistent pesticides such as atrazine, the enforcement of Cross-Compliance rules and the implementation of payment schemes such as Higher Level Stewardships. This is leading to a greater awareness of best agricultural practices (defined as ‘the equilibrium between economical, ecological and societal demands aimed to enhance sustainability of production systems’), which include the use of practices from both organic and conventional farming. Examples of this are schemes like LEAF (Linking Environment And Farming). Waitrose, one of the major UK supermarket chains, buys the majority of its fruit and vegetable stock from LEAF certified farms. The results presented in this thesis indicate that, at least in the system selected for study, plants grown in FYM amended soil showed increased growth and decreased stress when compared to plants grown in conventionally amended soil. However, it remains to be seen if best practice agriculture can deliver these benefits, while maintaining high yields.

7. 1. Future Work.

- Expand the Structural Equation Model with larger data sets including soil and plant mineral levels. Identify individual bacterial species, which show response to soil amendment by DGGE and DNA sequencing and then use Quantitative PCR methods to track their population density over the course of the experiment.
- Re-analyse volatile chemical emission from *A.thaliana* under insect herbivory and identify the type and levels of the chemicals present. Use

a plant species with relevant agronomical value (for example, *Vicia faba* or *Triticum aestivum*) and their major insect predators. However, it would be important to select a crop whose genome has been fully annotated, or is sufficiently similar to one that has.

- Identify differentially regulated proteins by MALDI-TOF MS that are currently un-identified.
- Perform a more detailed mineral analysis of *A.thaliana* grown on soil from the Palace Leas experimental hay plots, including both major mineral nutrients (sulphur, potassium, phosphorus, nitrogen and carbon) and trace elements (calcium, zinc, magnesium, sodium and boron). Perform a choice experiment with an insect pest on plants with "optimal" or "sub-optimal" mineral levels as according to the mineral balance hypothesis and the trophobiosis theory (Chabassou, 1985, Phelan et al 1996) to identify feeding preference.
- Test how other environmental factors, such as pH and mineral levels, affect the microbial community structures in soil from Plots 2 and 13. Establish whether the observed significant differences caused by the laboratory addition of mineral fertilisers are transient.

7. 2. References

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