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Article

Effects of Previous Crop Management, Fertilization Regime and Water Supply on Potato Tuber Proteome and Yield

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Abstract: There is increasing concern about the sustainability and environmental impacts of mineral fertilizer use in agriculture. Increased recycling of nutrients *via* the use of animal and green manures and fertilizers made from domestic organic waste may reduce reliance on mineral fertilizers. However, the relative availability of nutrients (especially nitrogen) is lower in organic compared to mineral fertilizers, which can result in significantly lower yields in nutrient demanding crops such as potato. It is therefore important to gain a better understanding of the factors affecting nutrient use efficiency (yield per unit fertilizer input) from organic fertilizers. Here we show that (a) previous crop management (organic *vs.* conventional fertilization and crop protection regimes), (b) organic fertilizer type and rate (composted cattle manure *vs.* composted chicken manure pellets) and (c) watering regimes (optimized and restricted) significantly affected leaf chlorophyll content, potato tuber N-concentration, proteome and yield. Protein inference by gel matching indicated several functional groups significantly affected by previous crop management and organic fertilizer type and rate, including stress/defense response, glycolysis and protein destination and storage. These results indicate genomic pathways controlling crop responses (nutrient use efficiency and yield) according to contrasting types

and rates of organic fertilizers that can be linked to the respective encoding genes.

Keywords: 2D-electrophoresis; chicken manure pellets; cattle manure; fertilization regime; potato; protein profile; *Solanum tuberosum*; water use

1. Introduction

There is increasing concern about the sustainability of intensive crop production systems and in particular their dependence on mineral (NPK) fertilizer inputs [1]. The manufacture of mineral N-fertilizer requires large energy inputs and is estimated to account for up to 10% of greenhouse gas (GHG) emissions from agriculture globally [2]. The high mineral fertilizer inputs used in crops such as potato causes significant N and P losses (through leaching and run-off) from agricultural land, which contributes to pollution and eventual eutrophication of fresh water and marine ecosystems [1,3]. There is also growing concern about the future availability of P, K and all other minerals used as fertilizer that are mined from finitely available natural deposits. For example, currently known deposits of phosphorus are estimated to last for only 30–100 years, depending on the rate of usage [4–6]. This combined with the increasing cost of mineral fertilizers has resulted in a re-evaluation of using organic fertilizers (animal and green manures, and organic waste composts) to replace or minimize the use of mineral fertilizer [7–9].

The use of organic fertilizers can increase the organic matter/carbon content, structural stability, biological activity and invertebrate biodiversity of soils, and reduce nitrogen and phosphorus leaching/run off [7–11]. However, at the same total N-input level, yields were often found to be lower with organic fertilizers compared with standard mineral fertilization regimes. Current environmental regulations limit applications of organic fertilizers to the equivalent of 170 kg N ha^{-1} $year^{-1}$ in order to minimize nitrate leaching losses [12]. For potato, the use of composted cattle manure at this input level was shown to result in (a) reduced concentrations of certain mineral nutrients (especially N and K) in plant tissues and leaf chlorophyll levels and (b) changes in protein expression profiles in tubers and most importantly, there was an increased expression of proteins involved in stress regulation [13,14]. This is thought to be due to (a) the virtual absence of plant available forms of nitrogen (NH_4^+ and NO_3^-) in composted manure and (b) insufficient release of plant available nutrients via mineralization of organic matter in soils; mineralization of soils is positively correlated to soil biological activity which is affected by previous soil management (e.g. increased by regular organic fertilizer inputs) and soil water availability [15,16]. This view is supported by the finding that potatoes fertilized with chicken manure pellets (which have a higher content of plant available N than composted manure) achieved higher yields than those grown in composted cattle manure treated soils [17].

It was therefore hypothesized that the N-use efficiency (potato yield per unit N-input) from organic fertilizer may be increased by (a) growing potato crops in soils which had regular organic matter inputs in previous years, (b) switching to organic fertilizers with a higher content of plant available N (e.g., chicken manure pellets) and (c) by optimizing soil water content [18,19], However, there is a lack of published studies to test these hypotheses. The development of both innovative soil and crop

management practices and breeding strategies aimed at improving nutrient (especially N, P and K) use efficiency and crop yields from organic fertilizer requires a greater understanding of plant response at the molecular level. Protein profiling (proteomics) is a reliable technique for the detection of gene products expressed within plant tissue, enabling a comparison of differential expression in gene products across cropping systems [20,21]. This is a functional genomics approach that can identify proteins that are linked to causal genes, and enable the potential development of functional molecular markers for crop improvement in nutrient use efficiency [22].

In this study we assessed the effects of (a) contrasting organic fertilizers (composted cattle manure and composted chicken manure pellets), and fertilizer input levels (85 and 170 kg N ha^{-1}); (b) previous crop management (soils managed to organic or conventional farming standards for 4 years) and (c) contrasting water supply, on leaf chlorophyll content, tuber yield, N concentration, and protein profiles.

2. Materials and Methods

2.1. Experimental Design

In 2005, a factorial pot experiment comparing contrasting fertilization and watering regimes was conducted at Close House Experimental Station (University of Newcastle). Seed potatoes of the variety Santé were planted (in April 2005) in 40 liter pots (one tuber per pot) containing soil collected from a field previously managed to either (i) conventional or (ii) organic farming practices. The two soils were collected from adjacent fields both with uniform sandy loam, at Nafferton Experimental Farm (University of Newcastle). The conventionally managed field had previously been sown with winter oilseed-rape, treated with aldicarb (Temik) for the control of soil pests, and received mineral fertilizer inputs. The soil from the field managed to organic farming standards had been cropped with a grass/red clover ley for four years and received applications of composted cattle manure. For each soil type, a factorial experiment was designed to compare fertilization type (composted cattle manure and composted chicken manure pellets), fertilization level (85 kg N ha^{-1} and 170 kg N ha^{-1}, plus a control—0 N) and watering regime (restricted and optimized), producing 10 treatments per soil type (Table 1). The two fertilization types contained different amounts of P and K kg^{-1} N (Table 2). Plants were observed daily throughout the whole period of growth, and water was applied at the rate of 2 liters of water per application to individual pots for the optimum treatment whenever plants began to show the first signs of stress/wilting. For the restricted watering treatment, 1 liter of water per pot was applied when the optimum treatment plants were watered and observations confirmed that at the time that water was applied, these plants were more stressed than those receiving optimum watering. A randomized block design was used, with the treatments randomized within each of 4 replicate blocks and the experiment was repeated.

Table 1. Experimental design to illustrate the combination of watering regimes, previous crop management (soil from conventional or organic managed fields), and fertilization types and levels (low input = 85 kg N ha^{-1}; High input = 170 kg N ha^{-1}). Asterisks indicate the eight treatments from which tubers samples were analyzed by 2D gel electrophoresis.

	Restricted watering	**Optimum watering**
Conventional previous crop management		
Control	No fertilization	No fertilization *
Composted cattle manure	Low input	Low input
	High input	High input *
Composted chicken manure pellets	Low input	Low input
	High input	High input *
Organic previous crop management		
Control	No fertilization	No fertilization *
Composted cattle manure	Low input	Low input *
	High input	High input *
Composted chicken manure pellets	Low input	Low input *
	High input	High input *

Table 2. Inputs of major nutrients applied (kg ha^{-1}) in composted cattle manure and chicken manure pellets at two application levels: low input = 85 kg N ha^{-1}; High input = 170 kg N ha^{-1}.

	Composted cattle manure		**Composted chicken manure pellets**	
	Low input	High input	Low input	High input
C	931	1863	745.7	1491.4
Total N	85	170	85	170
NH_4^+	1.0	2.0	20.2	40.4
NO_3^-	1.4	2.8	0.35	0.70
Organic N	82.6	165.2	64.5	129.0
P	27.2	54.4	24.3	48.6
P_2O_5	62.6	125.2	57.4	114.8
K	79.7	159.4	48.7	97.3
K_2O	95.6	191.2	57.4	114.8

2.2. Chlorophyll Content

Leaf chlorophyll content measurements were estimated at two different times (24 July and 3 August 2005) using a SPAD meter (SPAD-502, Konica Minolta Sensing Inc.-Japan) to determine the nitrogen status of plant leaves on 24 June and 3 August. These dates corresponded to 41 days (BBCH scale foliar growth stage 55—inflorescence emergence, tuber growth stage 40—tuber formation) and 81 days (BBCH scale foliar growth stage 80—berry formation, tuber growth stage 46—main period of tuber growth) after emergence [23].

2.3. Yield Assessments

Tuber yield was assessed at the end of October. The number and weight of tubers in each of three size categories: <45 mm, 45–65 mm, and 65–85 mm and totals were recorded. Tuber dry matter content was determined on a composite, fresh sub-sample of tubers of approximately 150 g taken from the different size grades, which was dried in a forced-draught oven at 80 °C for 48 h. Tuber nitrogen composition was analyzed for total N by Dumas combustion according to the application notes provided by the instrument's producer (LECO TruSpec Automated C/N Analyzer, LECO Corporation, USA).

2.4. Protein Profiling

A subset of the treatments included in the pot experiments was selected for protein profiling of the potato tubers (Table 1). Only tubers from plants under the optimum irrigation regime were used. Three fertilization treatments were selected (0 N, cattle manure compost at 170 kg N/ha and composted chicken manure pellets at 170 kg N/ha) for plants grown in soils that were previously under conventionally management, while all five fertilization treatments were selected (composted cattle manure at 85 kg N ha^{-1} and 170 kg N ha^{-1}, composted chicken manure pellets at 85 kg N ha^{-1} and 170 kg N ha^{-1}, and the control—0 N ha^{-1}) for plants grown in soils that were previously under organic management. An average size tuber was randomly collected from each of the selected treatments, washed in double distilled water then sliced, lyophilized, and milled into a fine powder for protein extraction.

2.4.1. Protein Extraction and 2-DE

Proteins were extracted from approximately 100 mg freeze dried material (typically equivalent to 0.9–1.0 g fresh tissue) using a phenol extraction method as previously described [24]. Total soluble protein content was measured using a 2D-Quant kit (Amersham). For each sample, 200 µg was precipitated from the phenol extraction buffer using a 2D-Clean Up kit (Amersham). The protein pellet was then resolubilized in 85 µL De-Streak buffer with 0.5% pH 4–7 IPG buffer. Protein extracts from each sample within each treatment were then pooled to give a total protein load of 800 µg per 2-DE gel (biological replicates: 8, technical replicates: 6).

First-dimension isoelectric focusing was performed using 18-cm isoelectric focusing (IEF) strips (Amersham Biosciences) with a linear pH range of 4–7 in an Ettan IPGPhor isoelectric focusing system. The IEF strips were rehydrated overnight with total protein, then focusing was run using the following conditions: from 0 to 500 V in 1 h, from 500 to 1000 V in 1 h, from 1000 V to 8000 V in 3 h, finally 8000 V until 36,000 Vh. After focusing, the strips were stored at −70 °C then equilibrated at room temperature in equilibration buffer (6 M urea, 50 mM Tris-HCl, pH 8.8, 30% (v/v) glycerol, 2% (w/v) SDS) with 1% (w/v) DTT for 10 min. This buffer was decanted and replaced with equilibration buffer containing 2.5% (w/v) iodoacetamide for a further 10 min. The second dimension was run in the Ettan DALTsix system with 19 × 23 cm homogeneous 12% SDS-PAGE gels, according to standard protocols [25]. The gels were run with constant 25- to 30-mA current overnight. The gels were fixed and stained with colloidal coomassie G-250 (Candiano) overnight and destained in double

distilled water. Gels were scanned with a flatbed scanner to give 16 bit resolution images for subsequent analysis.

2.4.2. Image Analysis

The 2-DE gel images were analyzed using Progenesis SameSpots (Nonlinear Dynamics). Protein spot volumes were normalized using the total spot volume normalization method, whereby each spot on a gel image is expressed relative to the total volume of all spots on that image. This method of normalization is designed to minimize differences in spot volume caused by gel–gel variation. Overall, 302 distinct spots were matched across the 2-DE gels, and their normalized spot volumes exported from Progenesis for further statistical analysis.

2.5. Statistical Analysis

The effects of previous crop management regime, fertilization type and level, and watering regime on SPAD measurements, the potato tuber yield, and protein expression were assessed using ANOVA derived from linear mixed-effects models [26]. In an initial four factor ANOVA, SPAD data, tuber yield, tuber N concentration and percentage dry matter were analyzed with experiment, watering regime, soil type and fertilization regime as fixed factors, and experiment, replicate blocks, and watering regime as random factors [27]. We found that experiment was not a significant factor affecting any of the traits (data not shown). Therefore, the data was re-analyzed using 3 factor ANOVA (without experiment as a fixed factor). For the normalized protein spot data, the effects of soil and fertilization regime (at the high input level and optimum watering regime) were tested in a two way ANOVA with soil and fertilization regime as fixed factors, and replicate blocks as random factors. All analyses were carried out using the nlme library in the R statistical environment [28]. Residual normality was assessed using the qqnorm function in R [27], with no data showing serious violations from normality.

The relationships of tuber yield components and tuber protein spots to the agronomic regimes were investigated with redundancy analyses (RDA), using the CANOCO package [29]. Automatic forward selection of the agronomic regimes within RDA was used and their significance calculated using Monte Carlo permutation tests.

2.6. Protein Inference by Gel Matching

Protein spots whose abundance was significantly affected by Previous Crop Management (PCM), and/or fertilization type and/or rate were selected for inference by gel matching, in order to assign an inferred function. This method has been used previously to infer function based on gel matching in previous studies when protein identification was not possible [30]. Gel matching in the present study was based on a reference gel image, created by Progenesis Same Spots (Nonlinear Dynamics) during the image analysis procedure. The reference gel image was used to compare the migration of spots with three previously published potato tuber proteome maps that had been generated by the same 2-DE gel methods to separate protein spots [14,31,32]. Of these three studies, one of them used the same potato variety as used in the present study—Santé [14]. Proteins were inferred by matching their

spatial pattern between the reference gel image generated in this study to the published maps. A grid was created onto the reference gel image and published maps to aid gel matching. Protein spots had to meet several criteria to meet confidence of inference: (i) proteins spots were characteristic, *i.e.*, present on the reference gel image and had low variance within treatments, (ii) protein spots were one of several in an area of the gel that formed a characteristic pattern replicable over gels in the present study and the previously published gel map used for matching. Protein spots that did not meet these criteria were discarded. For many protein spots, there was a positive match between all three studies, giving us confidence in this gel matching approach. We have cited multiple references for protein spots that were matched and identified in multiple studies. However, a large proportion of the matched spots only have one or two of the three citations; in these cases the protein spots were only identified in one or two of the published studies even if they were matched across gel maps in all three studies. In the cases where we matched the same protein spot across two or three of the published maps and that had been identified in each study, the protein identifications were the same (*i.e.*, conflicting protein identities were not found), thus further increasing our confidence in this gel matching approach.

3. Results

3.1. Effects of Previous Crop Management, Fertilization Regime, and Watering Supply on Potato Agronomic Traits

3.1.1. Chlorophyll Content of Potato Leaves

SPAD measurements were taken as an estimate of chlorophyll content of potato leaves at 41 and 81 days after emergence; chlorophyll content is known to be closely linked to N status of potato plants. Chlorophyll content was lower at the later assessment date, 81 days after emergence compared to 41 days after emergence (supplementary Table S1).

Two factors in the analysis (watering regime and fertilization regime) significantly affected chlorophyll content at both dates, and previous crop management had a significant effect at 41 days after emergence ($p < 0.05$, supplementary Table S1). Chlorophyll content was significantly higher when the restricted watering regime was used ($p < 0.001$), and when plants were grown in PCM_{con} (conventional Previous Crop Management, at 41 days after emergence only, $p < 0.05$). Chlorophyll content was highest in soils fertilized with composted chicken manure pellets at the higher input rate (170 kg N ha^{-1}) followed by composted chicken manure pellets at 85 kg N ha^{-1} then the two cattle manure compost input treatments and lowest in the non-fertilized control.

A significant interaction between watering regime and previous crop management was detected at both dates ($p < 0.05$, supplementary Table S2). With restricted watering, potato leaves had a lower amount of chlorophyll when grown in PCM_{org} (organic Previous Crop Management) compared to PCM_{con}. In contrast, when the optimized watering regime was used, higher chlorophyll content was detected in plants grown in PCM_{org} when compared to plants grown in PCM_{con}.

3.1.2. Potato Tuber Yield

Total tuber fresh yield was significantly ($p < 0.001$) affected by each of the three experimental factors: previous crop management (soil previously managed to organic or conventional fertilization, crop protection and tillage standards), fertilization regime (type and level of fertilizer input applied before planting potato), and watering regime (restricted and optimized) (Table 3). Yield recorded for two of the three tuber size categories (65–85 mm and <45 mm) was also significantly affected by previous crop management, fertilization regime, and watering regime ($p < 0.05$, supplementary Table S3). In contrast, only previous soil management significantly affected dry matter content of tubers ($p < 0.0001$). Growing potatoes in soils that were previously under conventional management (PCM_{con}) led to significantly higher total tuber yield, but lower dry matter content in tubers when compared to potato grown in soils that were previously managed to organic farming standards (PCM_{org}) (Table 3). There were no effects of interactions between factors on tuber yield components.

Table 3. Effects of previous crop management (PCM), fertilization regime (FR), and watering regime (WR) on total tuber yield, and tuber N concentration.

Factor	Tuber number plant^{-1}	Tuber fresh weight (g) plant^{-1}	N concentration (%)	Dry matter (%)
Means (± SE)				
WR				
Restricted	13.2 ± 0.4	575.3 ± 10.9	1.16 ± 0.02	23.8 ± 0.2
Optimized	14.1 ± 0.3	733.4 ± 12.0	1.03 ± 0.02	23.1 ± 0.3
PCM				
Conventional soil	15.2 ± 0.4	691.5 ± 14.8	1.09 ± 0.03	22.7 ± 0.2
Organic soil	12.1 ± 0.3	617.1 ± 12.8	1.10 ± 0.02	24.1 ± 0.2
FR				
Control	12.3 ± 0.4 c	563.9 ± 17.6 d	1.02 ± 0.03	23.5 ± 0.4 ab
Composted cattle manure, 85 kg N ha^{-1}	12.9 ± 0.6 bc	620.0 ± 16.4 c	1.09 ± 0.04	23.4 ± 0.4 ab
Composted chicken manure pellets, 85 kg N ha^{-1}	13.2 ± 0.7 bc	667.7 ± 21.8 b	1.09 ± 0.04	23.6 ± 0.4 ab
Composted cattle manure, 170 kg N ha^{-1}	14.1 ± 0.5 b	654.6 ± 19.7 bc	1.14 ± 0.03	22.8 ± 0.5 b
Composted chicken manure pellets, 170 kg N ha^{-1}	15.7 ± 0.6 a	765.4 ± 22.9 a	1.14 ± 0.04	23.9 ± 0.3 a
ANOVA				
Main effects				
WR	ns	<0.0001	0.0024	0.0615
PCM	<0.0001	<0.0001	ns	<0.0001
FM	<0.0001	<0.0001	0.0607	ns
Interaction effects				
WR × PCM	ns	ns	0.0062	0.0777
WR × FR	ns	0.0711	ns	ns
PCM × FR	ns	ns	0.0452	0.0687
WR × PCM × FR	ns	ns	ns	ns

3.1.3. Nitrogen Content of Potato Tubers

The nitrogen content of tubers was significantly affected by watering regime (Table 3). Tuber N-content was significantly higher when the restricted watering regime was used ($p < 0.01$). There was also a significant 2-way interaction between watering and previous crop management (supplementary Table S2). When watering was restricted, tuber N concentration was higher in tubers grown in PCM_{con}. However, when watering was optimized, tuber N concentration was higher in tubers grown in PCM_{org} (supplementary Table S2). There was also a significant 2-way interaction between previous crop management and fertilization treatments (supplementary Table S4). In PCM_{con} the tuber N-content significantly increased when composted chicken manure input was doubled, while there were no significant differences between the two input levels in PCM_{org}.

3.1.4. Associations between Agronomic Factors and Tuber Yield and Yield Components

The biplot in Figure 1 shows the relationship between agronomic factors (mineral nutrient input from organic fertilizers, watering regime and previous soil management) and yield/yield components. Axis 1 explained most (39%) of the variation and axis 2 a further 3%. Most additional variance was explained by watering regime ($F = 36.7$, $P = 0.002$), available N input ($F = 26.1$, $P = 0.002$) and previous soil management ($F = 11.8$, $P = 0.002$), but C-input ($F = 12.5$, $P = 0.022$) and experiment ($F = 12.5$, $P = 0.004$) were also significant drivers.

Nutrient input levels (NH_4/NO_3-N, organic-N, P, C and K) and tuber yield components showed strong positive association along the positive axis 1 for the numbers and fresh weight of total and large tubers and weaker positive associations for the numbers and fresh weight of smaller tubers. In contrast, there were weak negative associations between nutrient input levels and dry matter and N concentrations in tubers along the negative axis 1 (Figure 2). Tuber yield components showed very similar associations with optimized watering regimes (WR2), previous conventional soil management (PCM_{con}) and experiment 2. Experiment 2 was also positively associated with leaf chlorophyll content (SPAD) along the negative axis 2.

Figure 1. Bi-plot derived from redundancy analysis showing the relationship between potato tuber yield components and agronomic factors (categorical variables: organic and conventional previous soil management (PCM_{org}, PCM_{con}), restricted and optimized watering regime (WR1, WR2), experiments 1 and 2 (Exp 1, Exp 2) indicated by black squares; continuous variables: C, P, K, organic-N [N_{org}], available N [N_{av}]). DM % = percentage dry matter of tubers, FW = tuber fresh weight and No. = number of tubers (in each tuber size category).

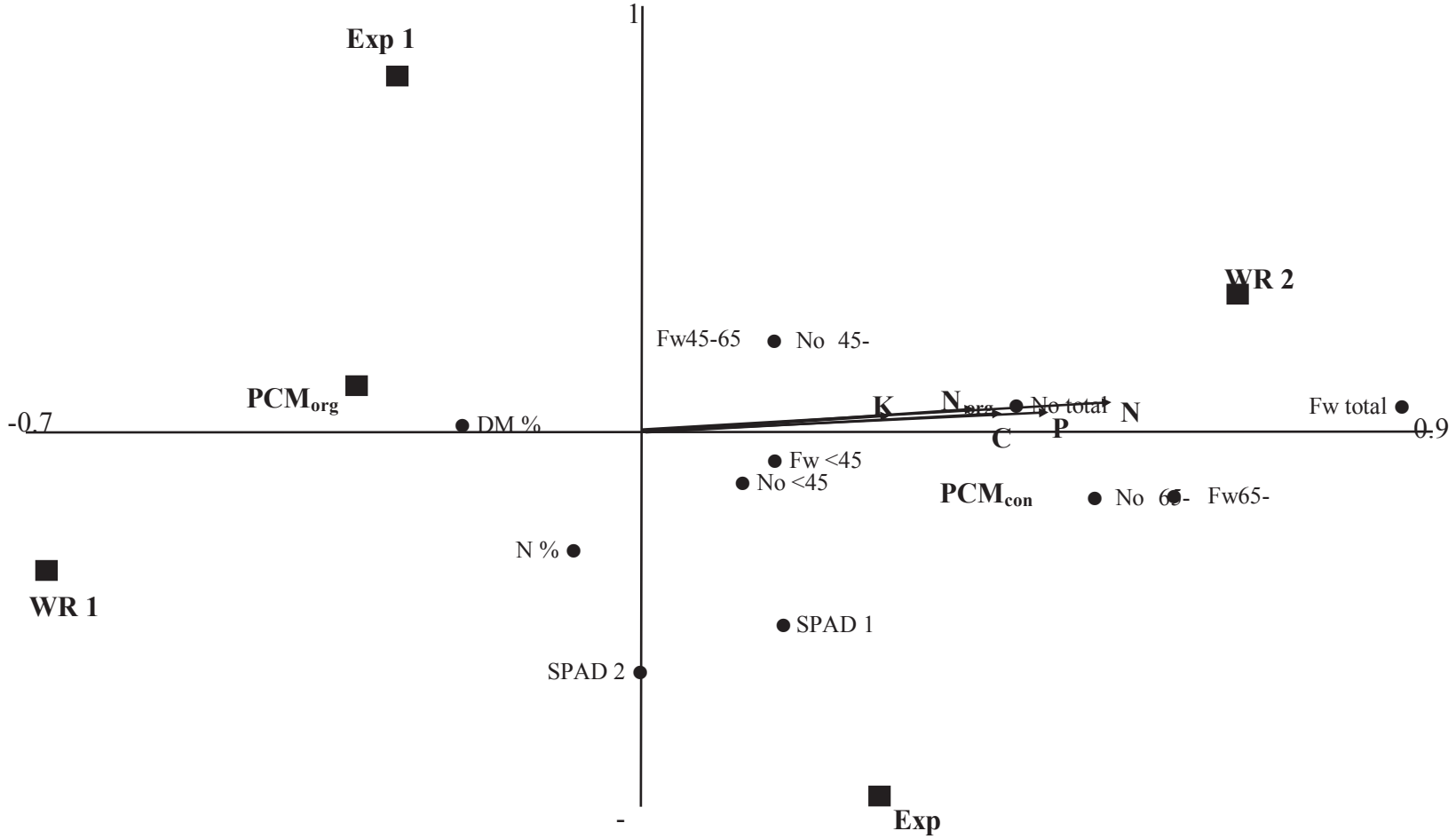
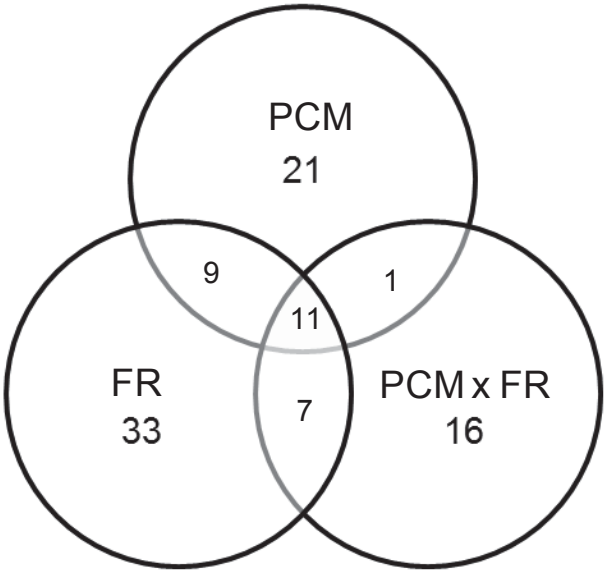


Figure 2. Venn diagram showing the number of protein spots significantly affected by previous crop management (PCM), fertilization regime (FR—composted chicken manure pellets *vs.* composted cattle manure at 170 kg N ha^{-1}), and the PCM × FR interaction effect ($p < 0.01$).



3.2. Effects of Previous Crop Management, Fertilization Regime, and Watering Supply on Potato Tuber Proteome

Potato tubers from a subsample of treatments within the optimized watering regime were used for proteomics analysis (Table 1). The selection of treatments enabled the effect of previous soil management and fertilizer inputs (but not watering regimes) on protein profiles (relative change in abundance of different proteins) in tubers to be determined. For the higher input level the effect of fertilizer input type was compared to the untreated control in both PCM_{org} and PCM_{con}. In addition we determined the effect of fertilizer input type and level in PCM_{org} only. A total of 302 protein spots were detected in the tuber proteome using 2D electrophoresis. Normalized spot volumes from each treatment and technical replicates were analyzed by ANOVA.

When the effect of fertilizer input type (composted chicken manure pellets and composted cattle manure applied at 170 kg N ha^{-1} or no input) and previous crop management (PCM_{org} and PCM_{con}) on the abundance of individual tuber protein spots was compared, a significant main effect of fertilization and/or previous crop management and/or a significant interaction between the two factors could be detected for 98 protein spots at the $P = 0.01$ level (Figure 2). Twenty one protein spots were significantly affected by previous crop management, 33 by fertilization regime, with 9 protein spots affected by both previous crop management and fertilization regime. A significant interaction between the two factors was detected for 16 protein spots (Figure 2). The associations between these protein spots and the agronomic factors included in the experiment were analyzed by RDA (see Figure 3 described below).

Figure 3. Bi-plot derived from redundancy analysis showing the relationship between protein spots and agronomic factors for (**A**) protein spots affected by fertilization regime (including those that were also affected by previous crop management); (**B**) protein spots affected by previous crop management (including those that were also affected by fertilization regime). Agronomic factors were: categorical variable, organic and conventional previous soil management, PCM_{org}, PCM_{con}, indicated by black squares; continuous variables, C, P, K, organic-N [N_{org}], available N [N_{av}]. Symbols indicate protein spots that had significantly increased abundance ($p < 0.01$) when no fertilizer (diamonds), high (170 kg N ha^{-1}) composted cattle manure (red triangles) or high (170 N ha^{-1}) composted chicken manure pellets (circles) was applied. The halos indicate protein spots that had significantly increased abundance ($p < 0.01$) when crops were grown in soils that were previously under either conventional crop management (black halo) or organic crop management (red halo). Transparent squares (Figure 3B only), correspond to protein spots shown by ANOVA not to be affected by fertilization regime.

Figure 3A:

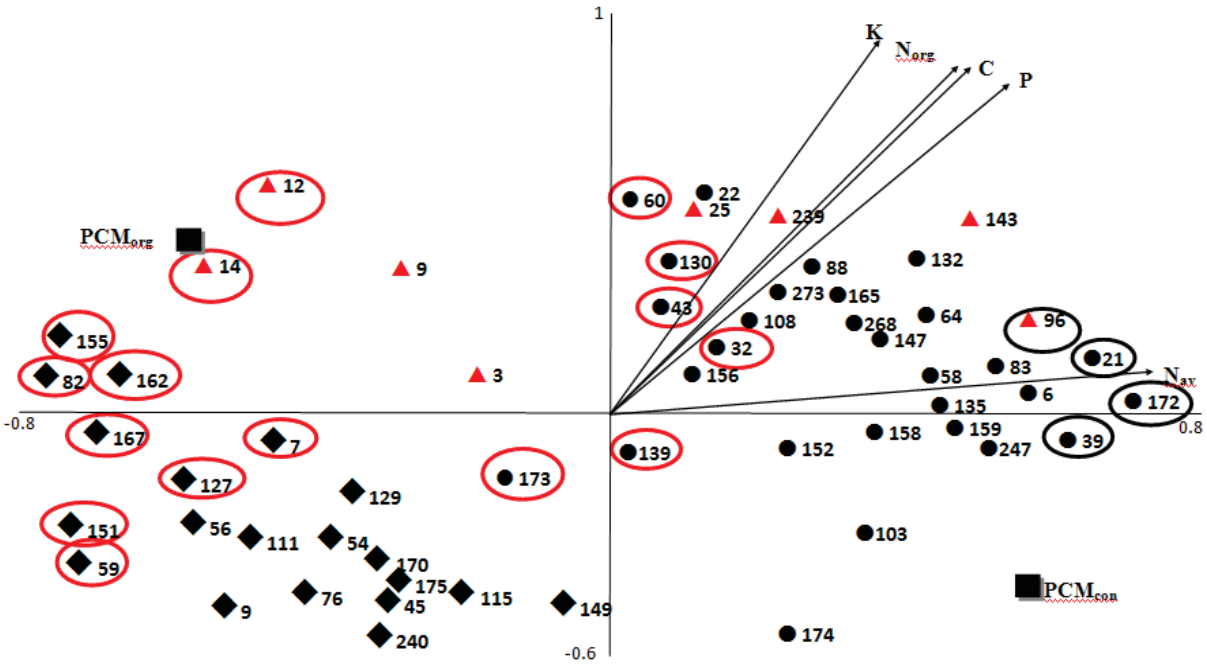
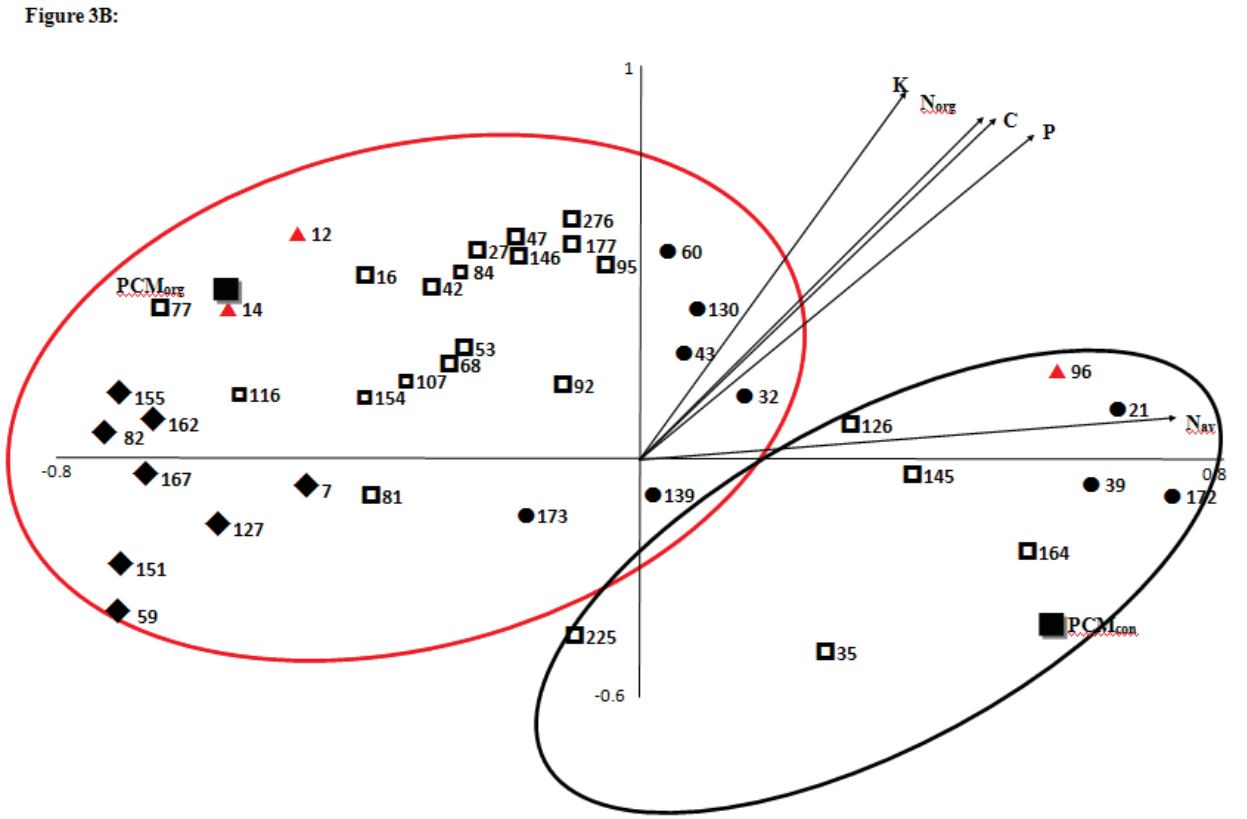


Figure 3. *Cont.*



When the effect of fertilizer type and input level was compared in only organic previous crop management (PCM_{org}) the abundance of 81 protein spots was significantly affected by fertilizer input type and/or levels at the $P = 0.01$ level. The associations between these protein spots and the agronomic factors included in the experiment were analyzed by RDA (see Figure 4 below).

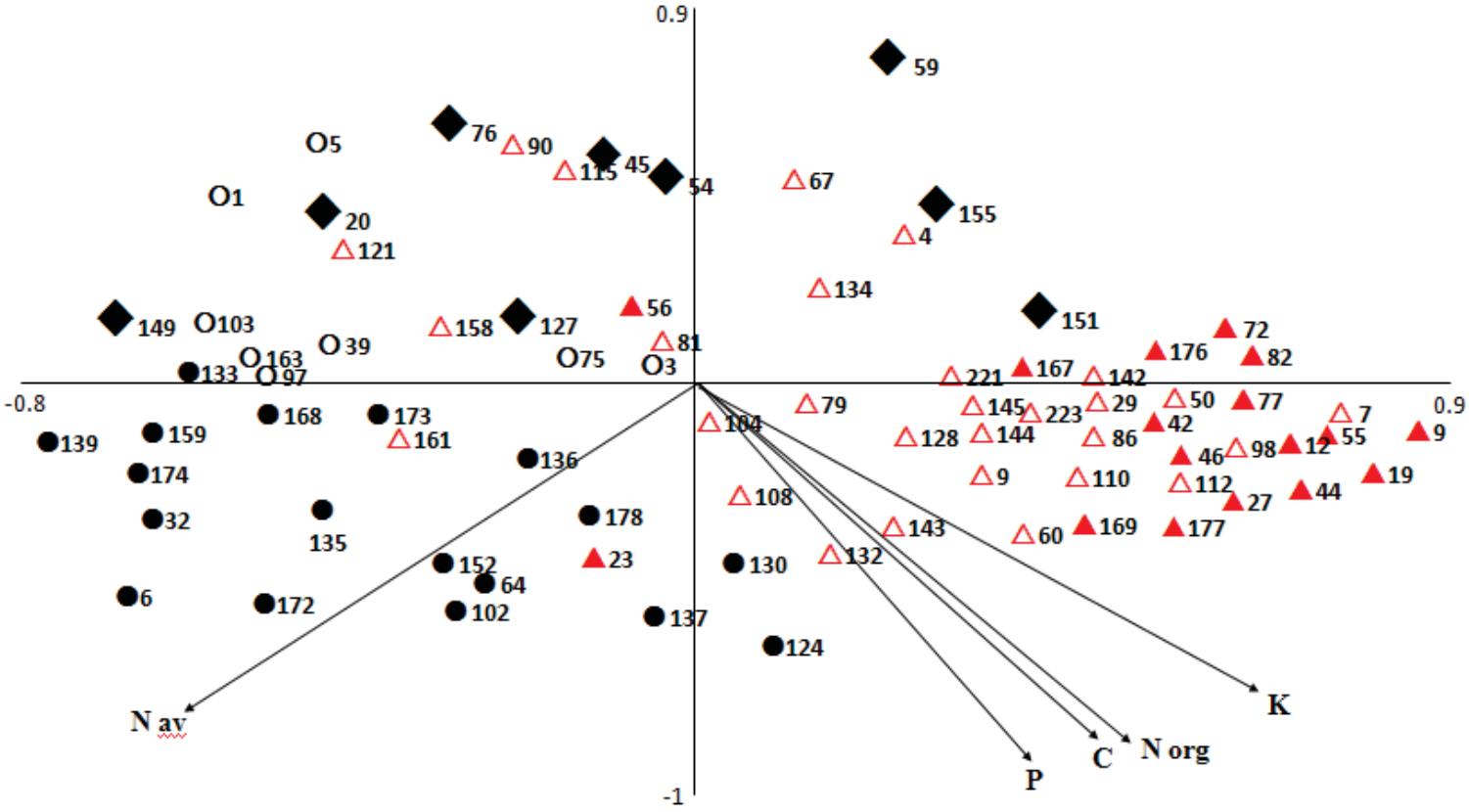
3.2.1. Associations between Agronomic Factors and Tuber Protein Profiles

Only proteins that were shown to be significantly affected by fertilization and/or previous soil management (Figure 2) were included in the RDA. The biplots in Figure 3 show the relationship between agronomic factors (mineral nutrient input from organic fertilizers and previous soil management) and proteins whose abundance was shown by ANOVA to be significantly affected by fertilization (Figure 3A) or previous crop management (Figure 3B). Axis 1 explained most (33%) of variation and axis 2 a further 5%. Most additional variance was explained by available N input ($F = 7.37$, $P = 0.004$), previous crop management ($F = 6.9$, $P = 0.004$) and C-input ($F = 2.5$, $P = 0.032$).

There were strong positive associations between nutrient input levels (NH_4/NO_3-N, organic-N, P, C and K) and (a) most proteins with increased abundance in tubers fertilized with composted chicken manure pellets and (b) some proteins (25, 96, 143, 239) with increased abundance in tubers fertilized with composted cattle manure along the positive axis 1. Similar associations along the positive axis 1 were detected for previous conventional crop management (Figure 3A,B). In contrast, negative association between nutrient input levels were detected for most proteins with increased abundance in tubers from non-fertilized plants and some proteins (3, 9, 12, 14) with increased abundance in tubers fertilized with cattle manure. Proteins 3, 9, 12 and 14 were also strongly positively associated with previous organic crop management along the negative axis 1 and the positive axis 2 (Figure 3A,B).

Four protein spots that were significantly affected by previous crop management, but not fertilization regime (Figure 3B) showed negative associations between protein abundance and nutrient input levels, but positive association with previous organic crop management. Only proteins 35, 126, 145 and 164 showed the opposite trend (Figure 3B). There were strong positive associations between nutrient input levels (NH_4/NO_3-N, organic-N, P, C and K) and most proteins with increased abundance in tubers fertilized with composted chicken manure pellets and some proteins (25, 96, 143, 239) with increased abundance in tubers fertilized with composted cattle manure along the positive axis 1 (Figure 3A). Similar associations along the positive axis 1 were detected for previous conventional crop management. In contrast, negative association between nutrient input levels were detected for most proteins with increased abundance in tubers from non-fertilized plants and some proteins (3, 9, 12, 14) with increased abundance in tubers fertilized with composted cattle manure. Proteins 3, 9, 12 and 14 were also strongly positively associated with previous organic crop management along both the negative axis 1 and the positive axis 2 (Figure 3A).

Figure 4. Bi-plot derived from redundancy analysis showing the relationship between protein spots and agronomic factors (categorical variable, organic and conventional previous soil management, PCM_{org}, PCM_{con}, indicated by black squares; continuous variables, C, P, K, organic-N [N_{org}], available N [N_{av}]). Symbols indicate protein spots that had significantly increased abundance ($p < 0.01$) when no fertilizer (diamonds), low composted cattle manure (transparent red triangles, 85 kg N ha^{-1}), high composted cattle manure (solid red triangle, 170 kg N ha^{-1}), low composted chicken manure pellets (transparent circles, 85 kg N ha^{-1}), or high chicken manure pellets (solid circles, 170 kg N ha^{-1}) was applied.



3.2.2. Associations between Fertilization (Types and Input Levels) and Tuber Protein Profiles

Only proteins that were shown to be significantly ($p < 0.01$) affected by fertilization in soils previously managed to organic farming standards were included in the RDA. The biplot in Figure 4 shows the relationship between nutrient input from organic fertilizers and proteins with significantly increased abundance with different fertilizer treatments (input types and levels). Axis 1 explained most (30.5%) of variation and axis 2 a further 3%. Most additional variance was explained by available N ($F = 6.82$, $P = 0.008$) and total K inputs ($F = 4.67$, $P = 0.012$).

There were strong positive associations between available-N (NH_4/NO_3-N) and most proteins with increased abundance in tubers fertilized with composted chicken manure pellets at the higher (175 kg ha^{-1}) input level along both the negative axis 1 and 2. Only 5 proteins (3, 75, 97, 103 and 163) showed significantly increased abundance at the lower (85 kg N ha^{-1}) composted chicken manure pellets input level, and these were positively associated with available N input along the negative axis 1 only. In contrast, most proteins with increased abundance in tubers fertilized with composted cattle manure at both the higher and lower input level were positively associated with K, C, P and organic N inputs along the positive axis 1 (Figure 4). All proteins with significantly increased abundance in tubers from non-fertilized control plants showed were negatively associated with nutrient inputs along axis 2.

3.2.3. Protein Inference by Gel Matching

Of the protein spots whose abundance was significantly ($p < 0.01$) affected by fertilization and / or previous crop management (PCM) treatments, the function of 47 was inferred by gel matching using 3 published potato tuber proteome maps [14,31,32] and the reference proteome map generated in this study (Figure 5). Proteins fell into six distinct functional groups: ATP synthesis/binding (2 protein spots), disease/defense/stress response (16 protein spots), energy production-glycolysis (11 protein spots), metabolism (3 protein spots), protein transport/destination/storage (11 protein spots), and protein breakdown (proteolysis/catabolism, 2 protein spots) (Table 4).

Some trends between the proteins inferred function and treatment that the protein spot was most highly abundant in were observed. Of the 16 disease/defense/stress response proteins, 8 had highest abundance in the composted chicken manure pellet (high rate) fertilization regime, whilst 3 had highest abundance in the control and 1 protein spot had highest abundance in the composted cattle manure (high rate) fertilization regime. Five protein spots were affected by PCM; 3 with higher abundance in PCM_{con} and 2 with higher abundance in PCM_{org}. An additional 3 defense related protein spots were affected by the fertilization × PCM interaction effect. Of the 11 protein spots with inferred roles in glycolysis, 6 had higher abundance in tubers from the composted cattle manure fertilizer regime; 3 protein spots in the low rate and 3 in the high rate of fertilizer input. Two glycolysis proteins had higher abundance in the tubers from the composted chicken manure pellets (high) fertilizer regime. Six protein spots were also affected by PCM: 4 with higher abundance in PCM_{org} and 2 with higher abundance in PCM_{con}.

Table 4. Abundance of protein spots (inferred by gel matching) that was significantly affected by fertilization (including protein spots affected by PCM (A), PCM (but not fertilization (B), a fertilization × PCM interaction effect (C).

Protein spot	Protein inference by gel matching	Function	Treatment with greatest protein spot abundance [1]	Reference protein spot [2]
A. Protein spots significantly affected by fertilization treatments (and PCM)				
176	Actin-54 (*N. tabacum*) Actin	ATP binding	Cattle (high)	4602 [31] 38 [32]
4	14-3-3 protein	Disease/defence	Cattle (high)	1315 [31]
23	Enolase	Energy-Glycolysis	Cattle (high)	4707 [31], 66 [32]
169	Enolase	Energy-Glycolysis	Cattle (high)	64 [32], 5711 [31]
2	Putative nascent polypeptide associated complex *a*-chain/expressed protein	Protein destination and storage	Cattle (high)	40 [32]
55	ATP binding/hydrolase/nucleosidetriphosphatase/ nucleotide binding (*A. thaliana*); Protein of AAA family (*C. annuum*)	Protein synthesis, storage and turnover	Cattle (high)	3827 [14]
177	Enolase	Energy-Glycolysis	Cattle (high), PCM_{org}	14 [32]
158	Malate dehydrogenase, cytosolic; Glyceraldehyde 3-phosphate dehydrogenase	Energy-Glycolysis	Cattle (low)	7 [32]
98	Enolase-like	Energy-Glycolysis	Cattle (low)	4710 [31]
165	Enolase	Energy-Glycolysis	Cattle (low)	20 [32] 4710 [31]
60	UTP-Glc-1-*P* uridylyltransferase	Metabolism	Cattle (low), PCM_{org}	45 [32]
6	Kunitz-type enzyme inhibitor/S9C11	Disease/defence	Chicken (high)	17 [32], 1201 [31]

Table 4. *Cont.*

Protein spot	Protein inference by gel matching	Function	Treatment with greatest protein spot abundance [1]	Reference protein spot [2]
A. Protein spots significantly affected by fertilization treatments (and PCM)				
156	Ascorbate peroxidase	Disease/defence	Chicken (high)	42 [32], 6301 [31]
171	Proteasome subunit	Disease/defence	Chicken (high)	5209 [31]
91	Fructokinase	Metabolism	Chicken (high)	31 [32], 4423 [31]
147	Chaperonin 21 precursor	Protein destination and storage	Chicken (high)	76 [32]
163	Cys proteinase precursor	Protein destination and storage	Chicken (high)	7205 [31] 34 [32]
18	EST (similar to small heat shock proteins)	Stress response	Chicken (high)	4208 [31]
152	Pathogenesis related protein 10	Stress response	Chicken (high)	6101 [31]
178	Hsp20.1 protein (*L. peruvianum*)	Stress response	Chicken (high)	4102 [32]
17	Ascorbate peroxidase	Disease/defence	Chicken (high), PCM_{con}	6304 [31]
21	Ascorbate peroxidase	Disease/defence	Chicken (high), PCM_{con}	5308 [31], 67 [32]
39	Triosephosphate isomerase, cytosolic isoform (*S. chacoense*)	Energy-Glycolysis	Chicken (high), PCM_{con}	6206 [31] 6 [32]
173	Glyceraldehyde 3-phosphate dehydrogenase	Energy-Glycolysis	Chicken (high), PCM_{org}	7506 [31] 7511 [14]
43	UTP-Glc-1-*P* uridylyltransferase	Metabolism	Chicken (high), PCM_{org}	37 [32]
64	ATP synthase *b*-chain precursor, mitochondrial	ATP synthesis	Chicken (low)	36 [32]
76	Patatin precursor	Protein destination and storage	Control	2402 [14]
149	Patatin precursor	Protein destination and storage	Control	2603 [31]

Table 4. *Cont.*

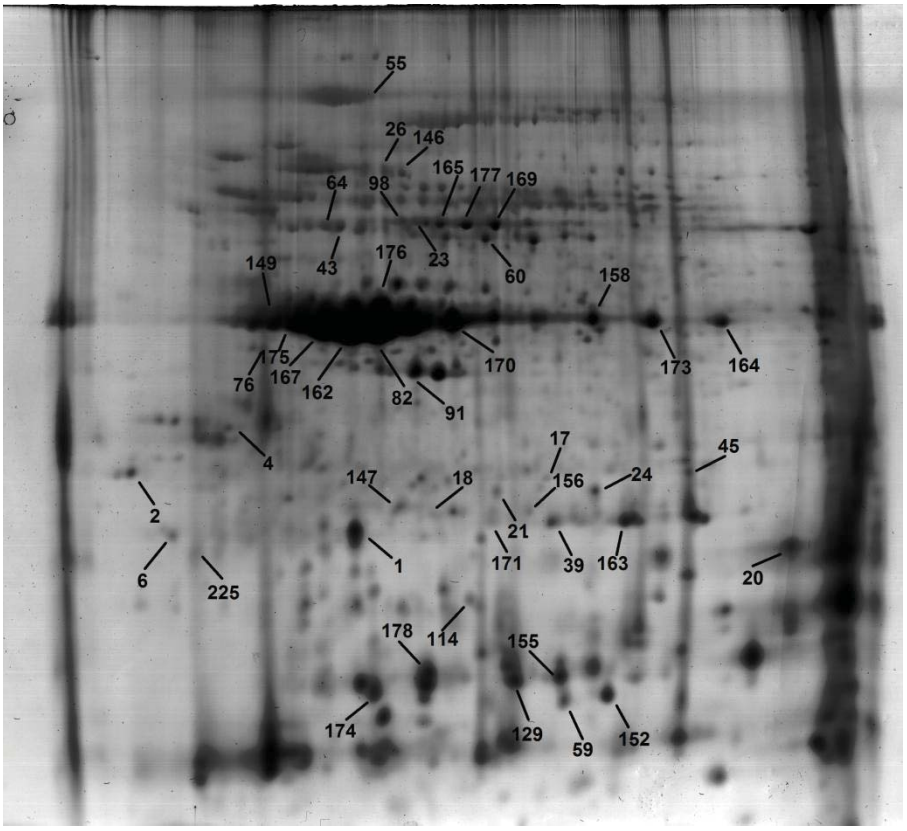
Protein spot	Protein inference by gel matching	Function	Treatment with greatest protein spot abundance [1]	Reference protein spot [2]
	A. Protein spots significantly affected by fertilization treatments (and PCM)			
170	Patatin protein 03 Patatin	Protein destination and storage	Control	4504 [31] 29 [32]
45	PRCI (*N. tabacum*) Proteasome subunit alpha type-6	ubiquitin-dependent protein catabolic process	Control	7209 [31]
20	EST (peptide sequences LGSHFVSENQDVSIK VAYSIVGPTHSPLR FSTSSSSTK YETGRPHSYK YETGRPHSYKLR IEKYETGRPHSYKLR)	Unclassified	Control	60 [32]
175	Patatin precursor; Patatin protein	Disease/defence Lipid degradation and metabolism,	Control	2610 [31]
59	Superoxide dismutase [Cu-Zn]	Disease/defence	Control, PCM_{org}	5 [32]
167	Patatin Patatin protein 03	Protein destination and storage	Control, PCM_{org}	26 [32] 3506 [31]
155	Hsp19.9 protein (*L. peruvianum*)	Stress response	Control, PCM_{org}	5108 [31]
	B. Protein spots significantly affected by PCM (not fertilization)			
225	Kunitz-type enzyme inhibitor S0C11	Disease/defence	PCM_{con}	3 [32]
164	Glyceraldehyde 3-phosphate dehydrogenase	Energy-Glycolysis	PCM_{con}	8403 [14] 8508 [31] 1 [32]
276	Phosphoglycerate mutase	Energy-Glycolysis	PCM_{org}	4809 [31]
146	Phosphoglycerate mutase	Energy-Glycolysis	PCM_{org}	4813 [31]

Table 4. *Cont.*

Protein spot	Protein inference by gel matching	Function	Treatment with greatest protein spot abundance [1]	Reference protein spot [2]
C. Protein spots significantly affected by fertilization × PCM interaction effect				
1	Transporter	Protein transport	PCMcon: cattle (high) PCM_{org}: chicken (low)	3209 [31]
24	Putative proteasome 20S beta1 subunit (*B. napus*)	Proteolysis	PCM_{con}: cattle (high) PCM_{org}: control	7202 [31]
174	Pathogenesis-related protein 10 (*S. virginianum*);	Disease/defence	PCM_{con}: control PCM_{org}: chicken (high)	3107 [31]
82	Patatin; Phosphoenolpyruvate carboxykinase	Protein destination and storage; signal transduction	PCM_{con}: control PCM_{org}: cattle (high)	28 [32], 4501 [31]
162	Patatin; Phosphoenolpyruvate carboxykinase Patatin protein 07	Protein destination and storage; Signal transduction Protein destination and storage	PCM_{con}: control PCM_{org}: cattle (high)	27 [32] 3509 [31]
114	Aspartic proteinase inhibitor 11 (AllergenSola t 2)	Stress response	PCM_{con}: control PCM_{org}: cattle (high)	4105 [31]
129	Pathogenesis related protein 10 Pathogenesis-related protein STH-2	Stress response Disease/defence	PCM_{con}: control PCM_{org}: cattle (high)	5103 [31] 2 [32]

Notes: [1] The category of treatment is listed according to the factor that significantly affected protein spot abundance (*i.e.*, organic or conventional Previous Crop Management—PCM_{org} or PCM_{con}; fertilizer—control (no fertilizer), chicken [composted chicken manure pellets, low (85 kg N ha^{-1}) or high (170 kg N ha^{-1})] or cattle [composted cattle manure low (85 kg N ha^{-1}) or high (170 kg N ha^{-1})]. Where fertilizer and PCM were both significant, the category of fertilizer is listed first. In cases where there was a significant fertilizer × PCM interaction, the category of fertilizer is listed for each PCM; [2] The protein spot number and published reference (in brackets) used for protein inference by gel matching.

Figure 5. 2D reference gel image of potato tuber proteome used for inference of protein function by gel matching to published potato tuber proteome maps.



4. Discussion

4.1. Effects of Previous Crop Management, Fertilization Regime, and Watering Supply on Potato Traits

Reducing the reliance on mineral fertilizers by increased use of organic fertilizers such as manure and composted organic waste (including sewage) is thought to be an important strategy to improve the sustainability of crop production and future food security (1,2,3). However, replacing mineral fertilizers with composted cattle manure was previously shown to significantly reduce yields and affect gene and protein expression pattern in potato, even when the maximum input level permitted under current Environmental legislation (170 kg ha^{-1} $annum^{-1}$) is used [13,14,33]. This is thought to be mainly due to insufficient supply of nitrogen from organic fertilizers, such as composted manure, which contain very low concentrations of water soluble, readily plant available forms of nitrogen (NO_3^-, NH_4^+). However, the omission of chemosynthetic pesticides in organic potato production also contributes to the yield differential between organic and conventional crops (18). Results reported here show that the use of composted chicken manure pellets (which have a higher concentration of readily plant available nitrogen) will significantly increase potato yields compared to cattle manure compost at the same total N-input level. The use of composted chicken manure pellets and/or composts supplemented with organic fertilizers with high plant available-N content (e.g., manure slurry or

vinasse, a by-product of the sugar industry) may therefore significantly increase yields in organic and other sustainable farming systems which aim to reduce mineral fertilizer inputs.

Nitrogen supply from composted manure to crops is reliant on microbial mineralization processes in soil and the total amount of nitrogen becoming available from fertilizer applied prior to the planting is known to be lower when organic rather than mineral fertilizers are used (12,18). Soil microbial activity (and associated mineralization capacity) was shown to increase in soils which receive regular organic fertilizers over long periods of time and both soil microbial activity and plant growth are reduced by suboptimal irrigation/water availability in soils (12, 14, 16). Potential strategies to improve crop yields from organic fertilizer inputs therefore include (a) the use of organic fertilizers with a higher content of available forms of N (e.g. chicken manure), (b) optimization of water supply and (c) more frequent use of organic fertilizer inputs (to increase mineralization capacity in soils). The higher levels of chlorophyll in leaves of plants fertilized with composted chicken manure pellets and RDA results observed in this study suggest that the use of composted chicken manure pellets (which contain 20 times higher concentrations of readily plant available N in form of NH_4^+) increased crop yield mainly *via* increasing N-supply to crops. N-supply is an important factor affecting photosynthesis with N deficiency reducing chlorophyll synthesis and growth of photosynthetic plant organs (stems and leaves) and resulting in early senescence [34]. Composted chicken manure pellets may also have stimulated mineralization, since previous studies showed poultry manure (but not cattle manure) inputs increases populations of bacterial feeding soil nematodes, which indicates increased microbiological activity and nutrient cycling [35].

In this study, previous organic crop management (which involved regular composted manure inputs over a 4 year period) reduced potato yields, when compared to conventional previous soil management (which involved regular mineral fertilizer inputs). This was unexpected, because previous comparative studies reported that replacing mineral fertilizer with manure inputs will result in an increase in soil microbiological activity and diversity after only two years [18,19]. Soil biological activity/ mineralization capacity was not assessed in this study, but results suggests that previous conventional crop management/mineral fertilization resulted in higher residual soil nutrient levels and that this had a greater effect on nutrient supply and crop yields than possible effects of regular organic fertilizer inputs on soil biological activity/mineralization capacity [11]. Further studies should therefore focus on quantifying the effect of previous crop management practices on microbial activity/mineralization capacity, soil nitrogen cycling and crop performance.

As expected, restricted watering resulted in lower potato tuber yield. However, when compared at the same fertilization regime, restricted watering resulted in a higher leaf chlorophyll content (which is closely correlated to N-availability), suggesting that restricted water supply restricted potato yield primarily *via* direct effects on plant growth rather than indirectly *via* reducing soil mineralization processes and N-supply [36–38]. Water stress is known to result in higher stomatal resistance in plant leaves as a way to reduce water loss, but this limits CO_2 diffusion into the sub-stomatal cavities, thus limiting photosynthesis [34].

4.2. Effects of Previous Crop Management and Fertilization Regime on the Potato Tuber Proteome

Previous studies showed that a switch from mineral fertilizer to composted cattle manure inputs results in significant differences in protein expression pattern, with more than 20% of protein spots being differentially expressed. In contrast, the use of different crop protection regimes (use or non-use of chemosynthetic pesticides) can have a very limited impact on the potato proteome [14]. Here we demonstrated that both previous crop management (organic *vs.* conventional) and contrasting organic fertilizer input types (composted chicken manure pellets and composted cattle manure) can result in significant changes in protein expression. Results from the RDA suggested that a main driver for contrasting protein expression was the difference in available N supplied by different fertilization treatments. The abundance of some proteins was affected by both previous crop management (PCM) and fertilization regimes indicating that the impact of PCM on these proteins was linked to the differences in nutrient availability associated with PCM. Protein inference by gel matching showed that the up-regulation of functional groups (e.g., plant defense, glycolysis) was not restricted to a single plant treatment. Rather, different proteins within the same functional groups were differentially up-regulated due to contrasting PCM/fertilization treatments. For example, different stages of the glycolysis pathway were up-regulated in the different treatments. Since proteomics is a "snapshot" analysis (*i.e.*, it only shows the differences in plant responses at a given moment in time) this data indicates that there are differences in the rate or timing of glycolysis between the treatments leading to the observed differences in abundance of proteins involved in the different stages of glycolysis. For other functional groups (e.g., defence/stress and protein destination), the different proteins are not linked as part of a single pathway. Therefore, this indicates that there is a specificity in the function that each protein performs (and therefore the molecular activity occurring in the tubers), according to the plant environment (treatment) that it is up-regulated in. Lehesranta *et al.* [14] previously found that the use of organic (composted cattle manure) fertilizer led to a higher stress response in potato tubers compared to the use of mineral fertilizer. In this study, we found that contrasting types and levels of organic fertilizer can also affect the stress response in potato tubers. A higher number of protein spots with an inferred role in stress response had higher abundance in tubers of composted chicken manure fertilized plants. In studies such as this, where mass spectrometry based protein identification was not feasible, gel matching can be used to provide interesting inferences of what the protein function could be [30]. Results from gel matching should be treated with caution due to uncertainty that can arise when comparing the spatial pattern of protein spots on 2D gels, especially across different studies and/or plant genotypes/varieties/cultivars. Care should be taken to only match gels that were run using the same 2DE conditions, as this will drastically affect the spatial pattern of protein spots. Whenever gel matching is used, attention should be given to the criteria employed to accept/reject inferred protein function, as cases can arise where e.g. variability between gels could cause errors leading to increased likelihood of false inference.

The results presented here demonstrate our ability to quantify a molecular response to contrasting types and rates of organic fertilizers that is associated with genes involved in nutrient use efficiency. This is an important finding that indicates the importance of optimizing the constitution of organic fertilizers to reduce yield loss when they are used instead of mineral fertilizers. Further study will be required to extrapolate to other potato varieties, to investigate whether genetic variation influences

nutrient use efficiencies in contrasting types of organic fertilizers. The use of proteomics enables linkage between genomic pathways controlling crop responses to contrasting fertilization (nutrient use efficiency and yield) and the respective encoding genes [14,20,21,39], which in future studies may be used complementary to transcriptomics analysis [13,33] for understanding gene expression pathways. This will facilitate the development of functional molecular markers to accelerate future crop breeding programs [22] that seek to improve crop yields and quality from organic fertilizers based on animal and green manures and recycled domestic organic waste (including sewage). Such strategies may be crucial to improve the sustainability, resource use efficiency and environmental impacts of crop production [1,3–6].

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