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Quantitative proteomics to study the response of potato to contrasting fertilisation regimes

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Abstract

There is concern over the sustainability and environmental impact of mineral fertilisers and crop protection inputs used in intensive arable crop production systems. However, replacing mineral with organic fertilisers (animal and green manures) and restricting the use of chemosynthetic crop protection may significantly reduce crop yields. The effects of (a) replacing mineral with composted cattle manure fertiliser input and (b) omitting pesticide-based crop protection on potato tuber yield, leaf and tuber mineral nutrient content and leaf protein profiles were investigated. Switching to organic fertiliser had a greater effect on yield and protein profiles than the omission of chemosynthetic crop protection. Leaf N and P composition were significant drivers of protein expression, particularly proteins involved in photosynthesis such as the large subunit of Rubisco, Rubisco activase and the photosystem I reaction centre, which were at higher abundance in potato leaves grown under mineral fertiliser regimes. Proteins known to be induced in response to stress, such as DHAR and GSTs were also shown to be up-regulated under mineral fertilisation, possibly associated with higher Cd composition, whereas two proteins known to be involved in biotic stress (1,3-beta-D-glucan glucanohydrolase; putative Kunitz-type tuber invertase inhibitor) were more abundant under compost fertilisation. Results showed that switching from mineral to organic fertilisers led to reduced N-availability, a significant change in leaf protein expression and lower tuber yield. In contrast, omission of chemosynthetic crop protection inputs had limited effects on protein expression and no significant effect on tuber yield. This study provides information on the effects of changes to nutrient supply on protein expression patterns. It is a pre-requisite for the development of functional molecular markers for a directed strategy to inform breeding programmes to improve potato nutrient use efficiency.

Introduction

The sustainability of arable crop production systems is of growing concern due to the increasing dependence on mineral fertiliser and crop protection inputs which are associated with significant negative environmental impacts (Tilman et al. 2002; Flynn and Smith 2010; Robertson and Vitousek 2009) and depletion of non renewable resources (Cordell et al. 2009; Fantel et al. 1985; Trehan and Sharma 2005). It is therefore important to focus on developing more sustainable fertility management practices to reduce the reliance on mineral fertilisers, whilst at the same time maintaining or improving current crop yield and quality. Legume fertility building crops, animal manures and composted domestic organic wastes are alternatives used in low input and organic agriculture (Munoz et al. 2005). For some crops/cropping systems they can produce similar yields when applied at the same total NPK input levels as mineral fertilisers (Hepperly et al. 2009; Herencia et al. 2007; Warman et al. 2009), and when organic fertilisers (e.g. composted manure) are used over longer periods of time they can lead to beneficial impacts on the soil, including increased soil fertility (Mäder et al. 2002; Eyre et al. 2009; Fliessbach et al. 2007; Herencia et al. 2007). They can also suppress soil borne diseases and/or induce disease, pest and abiotic stress resistance in plants (Ghorani et al. 2008; Giotis 2009), thus reducing the need for crop protection inputs.

The supply of N and other mineral nutrients from organic fertilisers is dependent on mineralisation of organic matter in the soil. Nutrient supply is therefore low immediately after planting in April/May and subsequently increases throughout the growing season as soil temperatures and mineralisation rates increase (Lammerts van Bueren et al. 2010). The low nutrient supply (especially of N) from organic fertilisers is a particular challenge for potato production, since modern, high yielding potato varieties are known to require approximately 2.5 to 5.9 kg/ha N, and 3.5 to 10.7 kg/ha K per tonne of tuber yield (Munoz et al. 2005). In a six year trial, yield of organically grown potato crops (cultivar 'Sante') were on average 40%

76 lower than conventional crops and this was in all years primarily due to insufficient N-supply
77 from organic fertiliser (composted manure) used in the organic system (QLIF 2009). In
78 contrast, there was no indication of P and K limitation in organically grown crops. Also less
79 efficient crop protection protocols used in organic systems (in particular for potato late
80 blight) contributed less to the yield differential and in 4 out of 6 seasons there was no
81 significant effect of crop protection. Furthermore, current legislation limits the application of
82 livestock manure to a maximum of 250 kg/ha N for a single crop and a farm average of 170
83 kg/ha N per year (The council of the European communities 1991). Phosphorus (P) and the
84 minor elements are required in relatively smaller quantities (White et al. 2008; Munoz et al.
85 2005). The nutrient use efficiency of potato plants is relatively low with less than 70% and
86 10% of N and P fertiliser respectively being recovered by plants. This is mainly due to
87 relatively small and shallow root systems, the fact that potatoes are often grown in sandy soils
88 and the limited arbuscular mycorrhizal development of modern potato varieties (McArthur
89 and Knowles 1992; Mäder et al. 2002; Westermann 2006). Thus the high nutrient demand of
90 the crop lead to higher nutrient inputs, nutrient losses and pollution (especially of N and P) in
91 potato compared to other arable crops.. There is therefore a need to breed potato varieties
92 with enhanced Nutrient Use Efficiency (NUE). The overall NUE of plants is determined by
93 two components which are its nutrient uptake efficiency (proportion of available nutrients
94 taken up) and nutrient utilisation efficiency (yield per unit nutrient taken up) (Dawson et al.
95 2008). In tuber crops such as potatoes, nutrients are initially utilised within foliar tissue to
96 drive processes such as photosynthesis. During the middle of the growing season (starting
97 during flowering), nutrients are remobilized and transported to the tubers, where they are
98 used to produce starch (Liu et al. 2008). Potato plants were shown to be less efficient in both
99 up take and utilisation of N, K and particularly P compared to other major arable crops
100 (White et al. 2008). Attempts to study the nutrient use efficiency of commercial potato

varieties indicate that potato varieties vary more in terms of N uptake capacity (ability to accumulate N within foliar tissue in the presence of abundant soil N) than in N-utilisation efficiency (Zebarth et al. 2004). Recent studies reported that protein and gene expression patterns in potato tubers were significantly affected by a switch from mineral to organic fertiliser inputs and associated reduction in N-supply (Van Dijk et al. 2012; Lehesranta et al. 2007). However, there is little information available on the responses in foliar tissues of potato resulting from a switch from mineral to organic fertiliser use and associated changes in nutrient supply pattern. Analysis of the leaf proteome has the potential to quantify the differential plant response to contrasting fertiliser regimes, and to identify the gene products, and hence pathways, involved (Good et al. 2004; Lehesranta et al. 2007; Kosová et al. 2011; Bahrman et al. 2004; Nawrocki et al. 2011).

The objectives of the study present were therefore to: (i) quantify the yield difference between contrasting fertiliser regimes (mineral NPK and composted cattle manure) and crop protection (chemosynthetic and no input) systems; (ii) quantify differences in compound leaf nutrient content as an indicator of nutrient availability under different management regimes, and (iii) identify the effects of contrasting fertiliser and crop protection regimes on leaf protein expression patterns. The underlying aim of the study was to improve our understanding of the responses of potato foliar tissue to nutrient limitation. Knowledge of these responses at the proteome level will aid the subsequent development of functional molecular markers for future targeted breeding/selection to improve nutrient use efficiency traits (e.g. QTLs linked to nutrient use efficiency molecular markers) in this major food crop. Such an approach is an important step towards sustainable crop production.

Materials and methods

Field design and experimental management

The Nafferton Factorial Systems Comparison (NFSC) Trial was established in 2001 on a field with a uniform sandy loam soil at Nafferton Farm (Newcastle University), near Stocksfield, Northumberland. The study consists of a series of four field experiments established within four blocks (replicates) where the effects of low input and conventional crop rotation, crop protection and fertility management practices were studied. Potato plants in this study were grown in 2009 and sampled from the organic rotation of experiment 4 of the NFSC, following a crop of winter wheat (year 2 of the rotation). The main plot factor was crop protection at two levels (low input and conventional) with fertility management (low input and conventional) as the subplot factor. Sub-plot size was 6 x 24 m. This allowed the experiment to be analysed as a 2 x 2 factorial design with identification of the effects of both fertility management and crop protection. The design generated 4 treatments: (1) compost fertility management (composted cattle manure) and ‘organic’ crop protection (mechanical weed control during ridging up and late blight controlled with copper fungicides); (2) compost fertility management and ‘conventional’ crop protection (usage of herbicides and fungicides according to British Farm Assured practice); (3) Mineral NPK fertilisation and ‘organic’ crop protection; (4) Mineral NPK fertility and ‘conventional’ crop protection. All treatments were replicated four times, generating 16 plots in total. Non-chitted seed tubers of the variety Santé were planted using a semi-automatic two-row potato planter. Potato seed tubers planted in plots treated with conventional crop protection were produced under conventional seed potato production conditions (Greenvale AP, Blairgowrie, UK). Potato seed tubers planted in plots treated with low input crop protection were produced under organic seed potato production standards (Greenvale AP, Blairgowrie, UK). All fertility

treatments were applied prior to planting of tubers and no irrigation was used (for a more detailed description of agronomic practices see online resource1).

Leaf sampling

Leaf samples for protein expression studies and mineral and heavy metal analysis were randomly harvested from each of the 16 plots at three different growth stages: inflorescence emergence (GS 59); flowering (GS 66); late flowering (GS 72) (Hack 2001), generating 48 samples in total. Forty terminal leaflets, each from the 5th compound leaf were collected (thus ensuring that leaves were of comparable age and position), immediately flash frozen with dry ice, and stored at -80°C for up to two weeks prior to freeze-drying. Freeze-dried leaves were milled (ultra-centrifugal mill, with a 0.2mm mesh screen, Retsch) and stored at -80°C until protein extraction.

Yield assessments and determination of NUE

After defoliation in late August, tubers were left in the ground for 4 weeks to allow skin maturation, and then harvested using a single-row potato harvester (Ransomes, Ipswich, U.K.). Potato yield was assessed by weighing the tubers from the two middle rows of each plot. Tubers from an individual plot were bulked together, placed in a 25kg bag for storage at ambient temperature for approximately 3 weeks and then graded. Randomly selected samples were used for nutrient composition analysis. Nutrient Use Efficiency (NUE) was calculated by dividing the dry matter yield with the total available N in soil during the beginning of the growing season (Zebarth et al. 2004).

Analysis of minerals and heavy metals in soil and leaves

In order to allow for within-plot variability, five cores of soil (0 to 30 cm) were randomly sampled within a plot and immediately mixed to form one composite sample per plot at three different time points: prior to planting (March 2009), during growth (end of June 2009) and after harvesting (October 2009). Soils were analysed for pH (1:1 in water), NO₃-N and NH₄-N, total organic C, N and Mehlich-3 extractable macro and micronutrients as detailed by Orr et al. (2011). This was conducted to show whether soil was deficient in nutrients prior to fertilisation, for example P availability in the soil was at index level 2 for both conventionally and organically fertilised soils which indicates that there was adequate P-availability in soil (Online Resource 2). Potato tubers and terminal leaflets were analysed for total N by Dumas combustion (LECO TruSpec Automated C/N Analyzer, LECO Corporation, USA). Other nutrients (P, K, S, Ca, Mg, Na, Fe, Mn, Cu, Zn, Mo, Cd, Al, B, Ni) were determined by subjecting the tubers and the terminal leaflets to acid digestion (H₂O₂, HNO₃) in a closed-vessel microwave reaction system (MarsExpress; CEM Corp., Matthews, NC, USA) and analysing with an inductively coupled argon plasma optical emission spectrometer equipped with a CCD detector (Vista-Pro Axial; Varian Pty Ltd, Mulgrave, Australia).

Protein extraction and 2D Difference Gel Electrophoresis (DIGE)

Proteins were extracted from freeze-dried leaf powder by TCA/acetone precipitation as follows. Freeze dried and milled potato leaf (1g) was added to 25ml of 10% TCA in acetone and left at -20°C for approximately 12 h to maximise protein precipitation. Following centrifugation at 12,000g the protein pellet was washed with acetone and 2-mecaptoethanol until decolouration (approximately 7 washes) and re-solubilised in DIGE sample buffer containing 7 M urea, 2M thiourea, 2% CHAPS, 1% protease inhibitor (GE Healthcare). Non-protein contaminants were removed from the samples using the Clean-Up kit (GE Healthcare), and subsequently resolubilised in sample buffer. The pH was adjusted to pH8.5

using pH10 DIGE sample buffer and subsequently quantified using the Quant kit (GE Healthcare). A total of 50µg of each protein sample was labelled with fluorescent CyDyes (GE Healthcare). Of the 48 samples (leaves collected from 16 plots x 3 growth stages), 24 were labelled with Cy3 and 24 labelled with Cy5. The samples were randomised, to randomly allocate dyes to samples, and sample to gels as recommended. An internal standard (comprising 25µg of each of the 48 samples) was labelled with Cy2. For each gel, one sample labelled with Cy3, one sample labelled with Cy5, and 50 µg of the Cy2 labelled internal standard were combined, giving a total of 24 gel samples. Single gel analyses were made from each biological sample, as recommended for DIGE. Samples were run in the first dimension on 18cm pH3-10 non linear strips (GE Healthcare) using an extended IEF protocol (30v active rehydration/10h, 500v/500vh, 1000v/800vh, 8000v/13500vh, 8000v/20000vh, 1000v/18000vh, 1000v/18000vh) followed by electrophoresis in the 2nd dimension SDS-PAGE 12.5% 2-D acrylamide gels.

Gels were scanned immediately after running using a DIGE scanner (GE Healthcare). Three images were generated from each gel (sample labelled with Cy3, Cy5, and the internal standard), generating 72 gel images. These images were analysed using Progenesis SameSpots (Nonlinear Dynamics). Overall, 259 distinct protein spots were matched across the gels. The normalised spot volume (the volume of each normalised to the corresponding spot volume in the internal standard, which was in turn normalised to a reference internal standard) was exported for further analysis by ANOVA.

Protein Identification

Protein spots that were significantly ($p < 0.01$) affected by crop protection or fertility management treatments, or the interaction between these two parameters were selected for identification. Spots were picked from preparative gels. All methods for preparative gels

corresponded to the method for DIGE ‘analytical’ gels (as described above), with two differences: (i) 800 µg protein was used for each preparative gel; (ii) preparative gels were post fixed (10% methanol and 7.5% acetic acid) overnight, stained with coomassie blue G-250 overnight, and then washed (7.5% acetic acid) to remove background stain. Spots were excised manually from preparative gels. Digest of spots with trypsin and protein identification of spots by MALDI-TOF was conducted by NEPAF (Cells North East Ltd., Newcastle upon Tyne). Protein Mass Fingerprints (PMF) were matched to SwissProt, uniprot.potato, tremble and NCBI (viridiplante) databases using a modified MASCOT search (Mascot parameters: Mass tolerance MS 0.1 Da, carboxamidomethylation of Cys as a fixed modification and Oxidation of Met as a variable modification). Proteins that could not be identified by PMFs were analysed by MALDI- MS/MS. MS/MS sequences were matched to NCBI (viridiplante) database.

Statistical analysis

The effects of fertiliser management, crop protection and their interaction (fertiliser x crop protection) on potato yield parameters, leaf protein expression and nutrient composition were analysed by ANOVA. Linear mixed-effects models (Pinheiro and Bates 2000) were used to analyse the data to produce ANOVA *P*-values for main effects and all interactions using (nlme package) R software (R Development Core Team 2009). The hierarchical nature of the split plot design was reflected in the random error structures that were specified as block/crop protection. The normality of the residuals of all models was tested using QQ-plots and data were cube-root transformed where necessary to meet the criteria of normal data distribution. The relationships of protein spots to leaf nutrients were investigated with redundancy analyses (RDA), using the CANOCO package (Ter Braak and Šmilauer 1998). Automatic

forward selection of the leaf nutrients within RDA was used and their significance calculated using Monte Carlo permutation tests.

Results and Discussion

The effect of organic and conventional cropping systems on potato tuber yield, mineral availability and nutrient use efficiency (NUE)

Two main components that contrast between organic and conventional cropping systems were compared: fertilisation management (mineral NPK vs composted cattle manure), and crop protection regime (conventional vs organic). There was a highly significant effect of fertilisation management on potato yield ($p < 0.01$). However, crop protection regime did not have a significant effect (Online resource 3), which was most likely due to low late blight and soil borne disease pressure/severity in 2009 (data not presented). Tuber yield of potato grown under mineral fertiliser was higher than under compost fertiliser, possibly due to the lower N availability in compost treated plots (soil available mineral N was 82 kg /ha) in June 2009 (Online resource 3). However, tubers grown under compost fertiliser had higher dry matter contents compared to tubers grown under mineral fertiliser (Online resource 3). These results are in agreement with previous studies where it has been reported that the use of compost fertilisation can reduce potato yields by up to 25% and increase the tuber dry matter percentage due to lower nutrient availability, especially at the beginning of the growing season (Lammerts van Bueren et al. 2010; Maggio et al. 2008). Finckh et al (2006) report that for a potential yield of 35 t/ha, potato plants need to take up 110–130 kg N/ha for canopy development prior to the start of tuber bulking (early summer).

Soil N analysis indicates that the total available soil N to compost fertilised plants was only 20-25% of that available to mineral fertilised plants (Online resource 3). This is likely to

have been a main factor that caused the reduced yields in compost fertilised potato crops. Yield data also reflects the mineral N results - yields were also slightly higher when conventional fertility treatment was combined with conventional crop protection. Weed competition may be a key factor here. There is also an interesting interaction ($p < 0.05$) between crop protection and fertility management. Lower levels of mineral N under conventional fertility treatment combined with organic crop protection could be due to increased weed growth in these plots, which reduces soil mineral N levels. Furthermore, in mineral fertilized plots the level of available soil N was higher than the level actually applied. In addition a relatively large difference in total N availability between crop protection regimes under mineral fertilization was observed (Online resource 3). We have monitored soil mineral N levels of potato plots in late June for several previous years and often see much higher levels of mineral N in the conventional fertility treatments (greater than amounts of N fertiliser added) compared with organic fertility treatments. Possible mechanisms for this are: (a) priming of soil organic N pools (for soil nutrition parameters see online resource 2) by addition of mineral N, as well as priming by root exudates from the growing potato plants in conventionally fertilised treatments, coupled with (b) immobilisation of mineral N in compost-treated plots.

The availability of other nutrients was estimated by analysing the composition in potato tubers and terminal leaflets. Tubers grown under mineral fertiliser had significantly higher concentrations of N, K, Mg, B, Cd ($p < 0.05$), and Cu, Zn and Mn ($p < 0.01$) than tubers grown under compost fertilisation. However there were no differences in P concentration in the tubers between fertiliser managements (Online resource 5). In potato leaflets, fertiliser management significantly affected the composition of all nutrients except for K, Na, Fe, and Cu (Online resource 6). At all the observed growth stages (inflorescence emergence, early flowering and late flowering) leaflets grown under mineral fertiliser contained significantly

($p < 0.01$) higher levels of N, P, and Cd and significantly ($p < 0.05$) lower levels of Mo compared to leaflets grown under compost fertiliser. However differences in composition of other minerals were dependent on growth stage. At inflorescence emergence (GS59), potato leaflets grown under mineral fertiliser had higher levels of Zn, Cd and Mn, and lower levels of Al compared to the compost fertiliser regime (Online resource 6). At early flowering (GS66), potato terminal leaflets grown under mineral fertiliser regimes had higher levels of Zn, Mn, and Ca, Ni and lower levels of B compared to the compost fertiliser regime. At late flowering (GS72), potato terminal leaflets grown under mineral fertiliser had higher levels of Mg, and lower levels of B, Ca, Al compared to the compost fertiliser regime. The finding of higher Cd content in mineral fertilised potato tubers and leaflets confirms previous studies that reported significantly higher Cd levels in mineral fertilised crops (Rossi et al. 2008) and linked these Cd inputs to the use of superphosphate fertiliser applications (Cooper et al. 2011).

Crop protection was also a significant factor affecting the composition of several minerals in potato leaflets. A significantly ($p < 0.01$) higher level of Cu was detected in potato terminal leaflets at all three growth stages when grown under organic compared to conventional crop protection, possibly due to the application of copper (as copper oxychloride) instead of synthetic fungicides (Online resource 6). The levels of Mn ($p < 0.01$) at inflorescence, Zn, Fe ($p < 0.001$) and Mn ($p < 0.05$) at early flowering, and Na ($p < 0.05$), Zn and Mn ($p < 0.01$) at late flowering was higher in terminal leaflets grown under organic compared to conventional crop protection. Furthermore, there were significant interactions between fertilisation and crop protection for Al ($p < 0.05$) levels at inflorescence and P, Mg ($p < 0.05$) and Ca ($p < 0.01$) at early flowering (Online resource 6).

Plants grown under compost fertiliser had a significantly ($p < 0.001$) higher nitrogen use efficiency (129kg/kg) compared with plants grown under mineral fertilization

(27.03kg/kg) (Online resource 3). However crop protection did not affect NUE. Reductions in N availability is known to be related to increased NUE (Zebarth et al. 2004). Sub-optimum N uptake can advance senescence at an earlier stage, shortening the period of photosynthesis, which consequently allows a longer period of N remobilization from leaves to developing tubers, resulting in higher nitrogen utilization efficiency (Zebarth et al. 2004)

Differential effects of organic and conventional cropping systems on the potato leaf proteome

In total, 2D DIGE reproducibly separated 259 protein spots (Figure 1). Direct comparison of the number of protein spots with other studies that analyse potato photosynthetic foliar tissue with 2DE separation can be misleading. Protein spot numbers may vary according to tissue type and growth stage. For example, previous studies have reported the presence of 125 protein spots in petiole tissue, 300 protein spots in shoot tissue, and up to 500 protein spots in potato leaves (Shweta Shaha et al. 2011; Keyvan Aghaei et al. 2008; Zhenyu Liu and Dennis Halterman 2009). However, each of these studies used tissue from plants at an early stage of crop development (prior to initiation of tuber formation), whereas in the present study, leaflets were taken from plants during tuber yield development. No other studies appear to have analysed potato leaf tissue at a later growth stage (i.e. during tuber development).

Of the 259 protein spots, the abundance of 172 protein spots were significantly ($p<0.05$) different between contrasting fertilisation and crop protection treatments. The majority of these proteins were significantly affected in their protein spot abundance by fertilisation management (164 protein spots), compared to crop protection regime (9 protein spots). The lower number of protein spots affected by crop protection regime may have been due to the low disease severity in the 2009 growing season (data not presented) when late

blight was virtually absent. Furthermore, an interaction between fertilisation and crop protection regimes significantly affected the abundance ($p < 0.05$) of 20 protein spots.

For 62 of the 172 protein spots identified above, fertilisation treatment had an effect at a higher significance level ($p < 0.01$). Nineteen of these protein spots were significantly up-regulated in potato terminal leaflets grown under compost fertilisation, and 43 protein spots were significantly up-regulated in potato terminal leaflets grown under mineral fertilisation. An interaction between fertilisation and crop protection regimes was detected for one of these proteins (spot 146). From these 62 protein spots, 41 could be identified by Peptide Mass Fingerprinting (PMF) and MS/MS (Table 1 a, b, and Online Resource 7). Higher identification rates have been hampered by the absence of a fully annotated potato genome and associated potato EST databases. These identified protein spots indicated that major plant processes such as photosynthesis/ metabolism, and general stress responses were affected by fertilisation treatment (reported below). Further protein spots were identified that do not have a known function. For example, one such protein was the pentatricopeptide repeat-containing protein (Table 1 a, b, and Online Resource 7).

Photosynthesis and metabolism

Photosynthesis and metabolism were represented by 7 different proteins identified in 15 protein spots. Thirteen of these protein spots were more abundant in leaves from the mineral fertilisation regime. Five protein spots (41, 292, 349, 386, 409) were identified as the large subunit of Rubisco while one protein spot (499) as Rubisco activase. These proteins, which were at higher abundance in mineral fertilised potato leaves at the late flowering stage, are all involved in the Calvin cycle of photosynthesis. Rubisco is a key enzyme in the stroma of chloroplasts, which catalyzes the photosynthetic carbon dioxide oxidation and photorespiratory carbon oxidation. It is composed of eight chloroplast-encoded large subunits

and eight nuclear-encoded small subunits (Desimone et al. 1998). The up-regulation of specific Rubisco subunits in terminal leaflets from mineral NPK fertilised plants was highly associated with greater N and P composition in these leaflets compared to those from compost fertilised plants (figure 2 and Online Resource 4). It is known that increased levels of P can result in higher uptake of N, and greater allocation of N to Rubisco in several plant species including crops (Osaki et al. 1993; Warren and Adams 2002) leading to enhanced photosynthetic and growth responses (Warren and Adams 2002). The activity of Rubisco activase, observed to be up-regulated in leaflets from mineral fertilised plants in this study (protein spot 499), is associated with photosynthetic activity via its interaction with Rubisco (Portis 2003). Furthermore, the higher abundance of ATP synthase (essential for energy production, protein spot 57) and the photosystem I reaction centre (protein spots 71, 98) in the same leaflets is likely to be due to the higher rate of metabolism and photosynthesis as indicated by the up-regulated proteins presented here.

Leaflets from plants grown with mineral fertilisers also had a higher abundance of serine hydromethyltransferase (SHMT) (spots 156 and 226) compared to the compost fertilised leaves. SHMT catalyzes the conversion of glycine to serine, CO_2 and NH_3 . As well as liberating NH_3 within mesophyll cells, this SHMT catalysed reaction is thought to be the exclusive source of photorespiratory CO_2 release (Deuce and Neuburger 1999), (Kopriva and Bauwe 1995). Therefore, the up-regulation of SHMT in the terminal leaflets from mineral fertilised plants observed in the present study indicates that these plants had a higher rate of photorespiration, and remobilisation of NH_3 .

Other photosynthesis proteins that were at higher abundance in leaves from mineral fertilised plants include oxygen evolving enhancer protein and a fructose – bisphosphate aldolase (protein spots 75, 90, 150, 584). These proteins were also identified in other protein spots that were at higher abundance in leaflets from compost fertilised plants

(protein spots 432, 552 respectively). This suggests that multiple copies of these proteins exist in the potato proteome, and the expression of each protein is influenced by the fertilisation regime. Three photosynthesis related proteins that were at higher abundance and only identified in leaflets from compost fertilised plants were the chloroplast manganese stabilising protein (protein spot 85), which is reportedly involved in photosystem II assembly, stability and photoautotrophy, a subunit of Rubisco small chain (protein spot 269) and thylakoid formation 1 (protein spot 92). It is known that the thylakoid formation 1 protein is required for the normal organization of vesicles into mature thylakoid stacks and ultimately for leaf development. (Wang et al. 2004) reports that in *Arabidopsis* plants the deletion of the chloroplast-localized thylakoid formation1 gene product leads to deficient thylakoid formation and multicoloured leaves.

A protein spot of digalactosyl diacylglycerol (DGDG, protein spot 238) was at higher abundance in plants grown with compost fertilization. This enzyme is a major galactolipid and together with monogalactosyldiacylglycerol (MGDG) constitutes up to 80% of total glycolipids, representing the bulk of chloroplast membranes. Kelly et al (Kelly et al. 2003) reports that DGDG has a crucial role in plant development and photosynthesis due to its role on the donor site of photosystem II through the binding of extrinsic proteins required for stabilization of the oxygen-evolving complex. The increased abundance of DGDG in potato leaflets from plants grown under compost fertilization in this study may have been due to significantly lower levels of both N and P in the plant leaves. Nitrogen limitation in *Arabidopsis* can result in an increase in DGDG (Gaude et al. 2007). Furthermore, phosphate limitation in plant foliar tissue is also known to result in the up regulation of DGDG due to phospholipid deprivation (Hartel et al. 2000; Kelly and Dormann 2002) as DGDG replaces phospholipids in plastidial and extraplastidial membranes (Sakurai et al. 2007). These results

420 are consistent with the lower N and P levels (Online Resource 5A-C) available to potato
421 leaves grown under composted cattle manure fertilisation.

422

423 *Plant stress response*

424 Response to stress was represented by 8 different proteins identified in 15 protein spots. Nine
425 of these protein spots were more abundant in samples obtained from the mineral compared to
426 the compost fertilisation regime. Plants are known to produce antioxidant enzymes such as
427 dehydroascorbate reductase (DHAR) and catalases for scavenging ROS in response to stress
428 (Vranova et al. 2002). One such protein identified as dehydroascorbate reductase (DHAR)
429 (protein spots 14, 53, and 236) was upregulated in plants grown under mineral fertilisation.
430 This particular protein is known to be expressed when plants are exposed to environmental
431 stresses such as heavy metals (e.g. Cd), due to the formation of reactive oxygen species. (Gill
432 and Tuteja 2010). Furthermore redundancy analysis (Fig. 2b-c and Online Resource 4)
433 showed a positive association of DHAR with Cd composition at inflorescence emergence
434 (GS59) and with Cd, P and N composition at early flowering (GS 66). The expression of
435 DHAR in leaves is responsible for regenerating ascorbic acid (ASH) from an oxidized state,
436 and maintains photosynthetic function by limiting ROS-mediated damage due to regeneration
437 of ASH (Chen and Gallie 2008). The up-regulation of DHAR in the present study is thus
438 consistent with the higher levels of Cd accumulation reported for those plants. Metal-induced
439 up-regulation of this enzyme has also been observed in several other proteomic studies
440 (Ahsan et al. 2009). Yin et al (Yin et al. 2010) report that modified tobacco plants over
441 expressing DHAR are tolerant to stress caused by heavy metals (e.g. Al) by the maintenance
442 of high ASH levels. These studies indicate that DHAR up-regulation in potato leaves in the
443 current study was due to the higher level of Cd in the mineral fertilised leaflets compared to
444 compost fertilised ones. This same reasoning may be applied to the observed up-regulation of

catalases (protein spots 103, 130, 146, 171 and 449), which were also positively associated with Cd composition (Fig 2a-c and Online Resource 4) (Scandalios 1994; Guan and Scandalios 2002; Cheruth et al. 2009). Various studies also report increased catalase activity in the root tissue of many crop species under Cd stress. In stressed plants Cd_2^+ replaces Ca_2^+ in the photosystem II reaction centre, causing the inhibition of photosystem II photoactivation (Mobin and Khan 2007; Singh et al. 2008; Hsu and Kao 2004; Khan et al. 2007). Glutathione S-transferases (GSTs) are enzymes that catalyze the conjugation of the tripeptide glutathione to hydrophobic electrophilic, and cytotoxic substrates (Marrs 1996). Plant GSTs have been extensively studied because of their ability to detoxify herbicides, and improve herbicide tolerance. Furthermore it is reported that GSTs are involved in numerous stress responses including pathogen attack (Karoline Hahn and Gunter Strittmatter 1994), oxidative stress, and heavy-metal toxicity (Marrs 1996). In this study GST (protein spot 209) was up regulated in mineral NPK fertilised plants at inflorescence emerge (GS59) and was positively associated with Cd composition (Fig 2a and Online Resource 4). A glycine rich protein (GRP) spot (spot 207), which was up-regulated in mineral NPK fertilised plants at late (GS72) flowering and was also positively associated with Cd composition (Fig 2c and Online Resource 4). GRPs are proposed to be part of the defence or repair system of plants (Mousavi and Hotta 2005); and their expression appears to be regulated by several forms of environmental stress such as light (Kaldenhoff and Richter 1989), NaCl (Mousavi and Hotta 2005), abscisic acid, and water (Didierjean et al. 1992). Hence it is reasonable to suggest that the regulation of GST and GPR was in response to the high Cd content and the generation of oxidative stress.

Alcohol dehydrogenase (ADH. protein spots 153 and 239) was up-regulated in leaflets from compost fertilised plants at early (GS66) and late (GS72) flowering, and was associated with Na composition in leaves (Fig. 2 and Online Resource 4). ADH is known to be involved in mediating stress responses in plant roots and can also complete the stress

perception pathway systemically, resulting in expression of ADH in shoot tissues (Dolferus et al. 1994; Peters and Frenkel 2004; Singh et al. 2010). (Dolferus et al. 1994; Chung and Ferl 1999). This suggests that the terminal leaflets from compost fertilised plants were receiving stress signals from the roots.

Another protein spot that was more abundant in plants from the compost fertilisation regime (spot 407) was identified as ycf2 protein. It is known to function in the reduction of mRNA synthesis and its activation can occur during biotic and abiotic stresses (Drescher et al. 2000). Two further protein spots were identified as biotic stress defence related proteins. These were protein spot 211, identified as 1,3-beta-D-glucan glucanohydrolase, which is involved in carbohydrate metabolic processes and has a role in the defence of plants against fungal infection (Beffa and Meins 1996) and protein spot 172, identified as a putative Kunitz-type tuber invertase inhibitor. Although these proteins are reportedly associated with the biotic defence response, this study indicates that they are also involved in plant response to abiotic stress. Other studies have shown that rice plants stressed with low N responded with both the up- and down-regulation of many disease and defence related transcripts (Xingming et al. 2006).

Conclusions

In the present study, fertilisation regime was shown to be the main factor that affected potato tuber yield, and leaflet protein abundance when comparing the effect of contrasting cropping systems. This is similar to the conclusion of a recent study that investigated the effects of cropping systems on the potato tuber proteome (Lehesranta et al. 2007). Analyses on soil available N, and leaflet and tuber mineral composition conducted in this study indicated that the affects of fertilisation regime on yield and protein abundance were the result of differences in the availability of multiple minerals, including lower levels of N and P in compost fertiliser compared to mineral NPK fertiliser. It is not possible to compensate for this by significantly increasing organic fertiliser inputs due to maximum input levels set by current EU-environmental legislation. Feasible strategies to increase N-supply from organic fertilisers are (a) to increase the mineralisation capacity in soils, (b) to use organic fertilisers with a higher available N concentration, such as those based on poultry manure and/or (c) to breed potato varieties/genotypes which have a higher N-uptake or utilisation efficiency. Understanding how potato plants respond to sub-optimal levels of nutrients is an important step towards understanding the plant response to nutrient limitation, which is a prerequisite to the ultimate goal of breeding crop varieties that are more nutrient use efficient. In this study, 63% of the protein spots separated by 2D DIGE were differentially expressed in response to mineral and compost fertilisation regime. Higher abundance of several proteins involved in photosynthesis and metabolism were observed in leaves from mineral fertilised plants, whilst two proteins (digalactosyl diacylglycerol, DGDG and the thylakoid formation1 protein) known to be involved in photosynthesis under nutrient limiting conditions were at higher abundance in compost fertilised leaves. Proteins such as DGDG and thylakoid formation1 may be essential to maintain plant growth and production of crop yield under sub-optimal nutrient levels. Several plant stress proteins were also identified, which indicated that plants grown

514 under compost fertilisation were stressed, and that the use of conventional crop protectants
515 led to Cd stress. Other studies that compare plant protein expression in organic and
516 conventional cropping systems have reported that fertility management practices can cause
517 differences in approximately 15% of the potato tuber proteome (Lehesranta et al. 2007).
518 Protein synthesis and turnover, carbon and energy metabolism as well as defence responses
519 were found to be positively affected by organic fertilisation of potatoes and the authors
520 suggest that organic fertilisation leads to an increased stress response in potato tubers
521 (Lehesranta et al. 2007). On the other hand Nawrocki et al (2011) reported only a 5%
522 difference in the proteomes of cabbage leaves and carrot roots when organic and
523 conventional systems were compared. The lower level of differential protein expression
524 between cropping systems reported by these authors could have been due to the lower
525 difference in crop yields and plant tissue nutrient composition, compared to the differences
526 reported in our study.

527 The present study provides novel information on elucidating the differences in the
528 potato leaf proteome due to contrasting fertilisation regimes. It is thus an important step
529 towards understanding the plant response to nutrient limitation, and hence our ultimate goal
530 of breeding crop varieties that are more nutrient use efficient. In this study, plant response to
531 contrasting fertilization regimes was studied in a single potato genotype in a single year to
532 initially demonstrate a cause and effect response of contrasting crop management regimes.
533 The data presented here demonstrates our ability to identify a molecular response to
534 fertilization that is associated with genes involved in NUE. However, given that a single
535 genotype was used we were not able to consider potato responses to contrasting fertilisation
536 regimes that may be influenced by genetic or annual environmental variation. The differential
537 expression of specific proteins in different genotypes, developmental stages and changing
538 environmental conditions has the potential to lead to identification of functional molecular

539 markers to aid potato breeding programmes to select for specific traits such as NUE. Future
540 steps towards attaining this goal should focus on testing a range of contrasting potato
541 varieties, using an integrated agronomic, physiological and genetic approach in order to
542 identify genetic variability that can be exploited in molecular breeding programmes targeted
543 to NUE varieties. Such improvement would reduce our dependence on mineral fertilisers that
544 have negative economic and environmental impacts, and importantly, reduce the usage of
545 finitely available (P, K) minerals, so contributing to more effective and sustainable systems
546 for potato production.

547

548

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Online Resource legends

Online Resource 1: Field trial agronomic management

Online resource 2: Means and SE for soil quality and nutrient status parameters

Online Resource 3: Means and ANOVA values for tuber yield and dry matter, soil available N and plant NUE (DM yield/Soil available N) across contrasting fertilisation and crop management.

Online Resource 4: Multivariate analysis of nutrient and proteomics data for terminal leaflets sampled at three growth stages: inflorescence emergence (GS59), early flowering (GS66), late flowering (GS72).

Online Resource 5: Means and ANOVA data for nutrient analysis of potato tubers. Asterix denote p-levels: $\ast=p<0.05$, $\ast\ast=p<0.01$, $\ast\ast\ast=p<0.001$; ns = non significant; T= $p=0.049-0.1$

Online Resource 6 A-D: Means and ANOVA data for nutrient analysis of potato terminal leaflets sampled at three growth stages: GS59, GS66, and GS72. Asterix denote p-levels: $\ast=p<0.05$, $\ast\ast=p<0.01$, $\ast\ast\ast=p<0.001$; ns = non significant; T= $p=0.049-0.1$

Online Resource 7: Additional data on protein identification search on Tremble, Swissprot and Uniprot databases.

Figure Legends

Figure 1: Reference gel image showing the position of 62 protein spots that were differentially expressed in response to fertilisation at a high significance level ($p<0.01$).

Figure 2: Multivariate analysis of nutrient composition and protein spots significantly affected by fertilisation at a) inflorescence emergence (GS59), b) early flowering (GS56), and c) late flowering (GS72).

Tables

Table 1 a. Proteins up-regulated in potato terminal leaflets in response to compost fertilisation, at three growth scores and sequence coverage is provided in online resource 7.

Rank	Common protein name	Function / metabolism
49	Pentatricopeptide repeat-containing protein At2g34370, mitochondrial	Unknown
85	Chloroplast manganese stabilizing protein-II (Fragment)	Photosynthesis Photosystem II stabilization
92	Thylacoid formation 1	Photosynthesis
153	Alcohol dehydrogenase 2	Oxidation-reduction process
172	Putative Kunitz-type tuber invertase inhibitor (Fragment)	Stress response
211	1,3-beta-D-glucan glucanohydrolase (Glucan endo-1,3-beta-glucosidase a)	Carbohydrate metabolic process
238	Digalactosyldiacylglycerol synthase 2, chloroplastic	Stress response
239	Alcohol dehydrogenase 2	Oxidation-reduction process
269	Ribulose biphosphate carboxylase, chloroplastic	Oxidation-reduction process Photorespiration Reductive pentose-phosphate cycle
407	Protein ycf2	ATP-binding Nucleotide-binding
432	Oxygen-evolving enhancer protein 1, chloroplastic	Photosynthesis Photosystem II stabilization
552	Fructose-bisphosphate aldolase	Glycolysis

Table 1 b. Proteins up-regulated in potato terminal leaflets in response to mineral NPK fertilisation, at three growth

identification scores and sequence coverage is provided in supporting information Table S6.

Rank	Common protein name	Function / metabolism
14	Dehydroascorbate reductase	Oxidation-reduction process
41	Ribulose biphosphate carboxylase large chain	Calvin cycle Carbon dioxide fixation Photorespiration Photosynthesis
53	Dehydroascorbate reductase (Fragment)	Oxidation-reduction process
57	ATP synthase subunit beta, chloroplastic	ATP synthesis
71	Photosystem 1 reaction centre	Photosynthesis
75	Oxygen-evolving enhancer protein 1, chloroplastic	Photosynthesis Photosystem II stabilization
81	Chymotrypsin inhibitor	Protease inhibitor
90	Oxygen-evolving enhancer protein 1, chloroplastic	Photosynthesis Photosystem II stabilization
98	Photosystem 1 reaction centre	Photosynthesis
103	Catalase (Fragment)	Oxidation-reduction process Response to oxidative stress
123	Pentatricopeptide repeat-containing protein At3g16610	Un known
130	Catalase isozyme 1	Oxidation-reduction process
146	Catalase isozyme 2	Oxidation-reduction process
150	Oxygen-evolving enhancer protein 2, chloroplastic	Photosynthesis
156	Serine hydroxymethyltransferase, mitochondrial	One-carbon metabolism

171	Catalase isozyme 2	Oxidation-reduction process
207	Glycine rich protein	Stress response
209	Glytathione S tranferase	Stress response
226	Mixture	One-carbon metabolism
	Serine hydroxymethyltransferase, mitochondrial	
	Catalase (Fragment)	Oxidation-reduction process
		Response to oxidative stress
236	Dehydroascorbate reductase-like protein	Oxidation-reduction process
292	Ribulose biphosphate carboxylase large chain	Calvin cycle
		Carbon dioxide fixation
		Photorespiration
		Photosynthesis
349	Ribulose biphosphate carboxylase large chain	Calvin cycle
		Carbon dioxide fixation
		Photorespiration
		Photosynthesis
379	6-4 photolyase	DNA repair
386	Ribulose biphosphate carboxylase large chain	Calvin cycle
		Carbon dioxide fixation
		Photorespiration
		Photosynthesis
409	Ribulose biphosphate carboxylase large chain	Calvin cycle
		Carbon dioxide fixation
		Photorespiration
		Photosynthesis
449	Catalase	Hydrogen peroxide catabolic

		process
		Oxidation-reduction process
499	Ribulose biphosphate carboxylase/oxygenase activase, chloroplastic	Photosynthesis
535	Cytochrome P450 86B1	Cell wall biogenesis/degradation
584	Fructose-bisphosphate aldolase	glycolysis

Notes

¹ Asterisk refer to the level of significance as tested in ANOVA within each growth stage

(*= $p<0.05$; **= $p<0.01$; ***= $p<0.001$; ****= $p<0.0001$).

² Proteins whose volume was influenced by an interaction effect ($p<0.01$) (fertilisation x crop protection).

³ Fold change in protein expression in leaflets from compost fertilised plants compared to leaflets from mineral fertilised plants (Table 1a), or in leaflets from mineral fertilised plants compared to leaflets from compost fertilised plants (Table 1b).

⁴ Proteins whose volume was influenced by an interaction effect ($p<0.05$) (fertilisation x crop protection).

⁵ Proteins whose volume was influenced by Crop protection ($p<0.05$)

Figure 1

Figure 2

Supporting information

Online Resource 1. Field trial agronomic management

Date	Treatments	Details
March 2009	Organic fertilisation	Application of composted cattle manure (170 kg N/ha)
April 2009	Conventional fertilisation	Fertilised with P and K(134 kg/ha P: 200kg/ha K)
	Conventional fertilisation	Fertilised with ammonium nitrate (34.5%N) (180kg N/ha)
	Organic seed	Planted with <i>Solanum tuberosum</i> var. Sante
	Conventional seed	Planted with <i>Solanum tuberosum</i> var. Sante
	Organic	Potato beds ridged up
	Conventional	Potato beds ridged up
	Convention crop protection (Weed control)	Sprayed with : Linuron 1.21 l/ha Sprayed with : Pendimethalin 1.81 l/ha
	Organic crop protection (Weed control)	Mechanical cultivation
May 2009	All	Beginning of emergence
June 2009	Organic crop protection (Late blight control)	Sprayed with : Copper 4.6 l/ha
	Conventional crop protection (Late blight control)	Sprayed with : Fubol Gold 1.9 l/ha
July 2009	Organic crop protection	Sprayed with : Copper 4.6 l/ha
	Conventional crop protection	Sprayed with : Shirlan 300ml/ha

	Organic crop protection	Sprayed with : Copper 3 l/ha
	Conventional crop protection	Sprayed with : Fubol Gold 1.9 l/ha
	Organic crop protection	Sprayed with : Copper 3 l/ha
	Conventional crop protection	Sprayed with : Shirlan 300ml/ha
	Conventional crop protection	Sprayed with : Fubol gold 1.9 l/ha
August 2009	Organic crop protection	Sprayed with : Copper 3 l/ha
	Conventional crop protection	Sprayed with : Shirlan 300ml/ha
	Organic fertilisation	Start of senescence <u>(11/08/2009)</u>
	Conventional fertilisation	Start of senescence <u>(20/08/2009)</u>
	Conventional crop protection	Sprayed with : Shirlan 300ml/ha
	Organic fertilisation	Haulm destruction (mechanical flailing)
	Conventional fertilisation	Haulm destruction (sprayed with : Reglone 2 l/ha)
September 2009	All	Yields assessed

Online resource 2: Means and SE for soil quality and nutrient status parameters

Factor	pH	N	C	C:N	P	K	Ca	S	Mg	Na	Fe
		(mg/g)	(%)	ratio	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)	(mg/kg)
Organic CP											
Compost	6.88	2.46	2.79	11.41	43.56	53.19	2138.97	13.31	213.2	19.8	352.38
Fertilized	±0.07	±0.03	±0.1	±0.54	±4.68	±5.65	±117.4	±0.66	±10.6	±1.61	±32.3
Mineral	6.73	2.51	2.85	11.39	47.45	49.55	2010.66	13.75	220.15	23.2	374.17
Fertilized	±0.13	±0.1	±0.1	±0.14	±2.09	±4	±102.6	±0.36	±11.4	±8.28	±34.2
Conventional CP											
Compost	6.91	2.53	2.84	11.27	39.29	46.11	2115.21	12.89	207.23	18.2	354.09
Fertilized	±0.11	±0.06	±0.07	±0.16	±7	±5.56	±90.23	±0.37	±8.3	±2.68	±23.6
Mineral	6.73	2.5	2.85	11.42	48.23	46.98	1991.84	13.27	206.87	15	357.32
Fertilized	±0.09	±0.09	±0.1	±0.49	±5.8	±3.4	±95.3	±0.2	±6.2	±1.15	±25.1

Online Resource 23: Means and ANOVA values for tuber yield and dry matter, soil available N and plant NUE (DM yield/Soil available N) across contrasting fertilisation and crop management

Factor		Yield	Dry matter	Available	Available	Total available	NUE	Formatted Table
		(t/ha)	(%)	NH ₄ (kg/ha)	NO ₃ (kg/ha)	N (kg/ha)	(kg / kg)	
ANOVA	Conventional CP							
	Mineral fertilised	46.96	20.48	31.61	331.4	363.01	27.03	
		±3.82	±0.73	±7.2	±12.9	±17.9	±3.7	
	Compost fertilised	37.11	23.35	2.35	69.95	72.3	129.04	
		±5.08	±0.69	±1.6	±8.5	±10.1	±27.1	
	Organic CP							
	Mineral fertilised	39.85	21.76	14.96	292.7	307.6	28.25	
		±1.48	±0.34	±6.3	±19.2	±13.7	±0.9	
	Compost fertilised	32.75	22.87	2.72	79.78	82.5	93.01	
		±2.39	±0.52	±0.9	±6.9	±7.8	±9.9	
Main effects								
Fertilisation (FE)	0.0038	0.0100	0.0048	>0.0001	>0.0001	0.0005		
Crop protection (CP)	ns	ns	ns	ns	ns	ns		
2-way interactions								
FExCP	ns	ns	ns	ns	0.0302			

Online Resource 34. Multivariate analysis of Nutrient and Proteomics data for compound

leaves sampled at three growth stages: GS59 (inflorescence); GS66 (early flowering); GS72

(late flowering)

		GS59	GS66	GS72
Eigen value	Axis 1	68.3%	63.9%	69.8%
(%)	Axis 2	9.5%	14.9%	7.4%
Drivers	N	1.000	0.006	1.000
(F, P)	P		0.259	0.054
	K	0.198	0.298	0.292
	S	0.178	0.016	
	Ca	0.158	0.220	0.178
	Mg	0.336	0.440	0.424
	Na	0.090	0.068	0.004
	Fe	0.454		0.032
	Mn	0.108	0.144	0.544
	Cu	0.054	0.432	
	Zn	0.384	1.000	0.570
	Mo	0.002	0.062	0.130
	Cd			0.184
	Al	0.168	0.070	0.392
	B	0.112	0.016	0.002
	Ni			0.022

Online Resource 45. Means and ANOVA data for nutrient analysis of potato tubers. Asterisk denote p-levels: *=p<0.05, **=p<0.01,

***=p<.001; ns = non signifiant; T=p=0.049-0.1

Factor	N	P	K	S	Ca	Mg	Na	Cu	Zn	Mn	Fe	B	Mo	Al	Cd	Ni
Means (± SE)																
Conventional CP																
Mineral	1.51	0.18	1.48	0.14	0.04	0.10	31.54	5.26	14.17	5.80	35.54	5.12	0.34	16.55	206.30	319.99
fertilised	±0.15	±0.01	±0.10	±0.005	±0.003	±0.008	±1.4	±0.2	±1.3	±0.3	±1.8	±0.1	±0.06	±0.6	±35.2	±30.7
Compost	1.14	0.18	1.20	0.13	0.03	0.08	48.24	4.87	12.07	4.66	32.85	4.53	0.45	19.48	147.64	298.18
fertilised	±0.07	±0.001	±0.01	±0.004	±0.001	±0.006	±14.6	±0.1	±0.5	±0.3	±2.7	±0.3	±0.06	±4.2	±10.4	±32.6
Organic CP																
Mineral	1.44	0.19	1.33	0.13	0.04	0.09	44.47	7.06	14.66	5.58	50.90	5.01	0.34	33.38	230.96	426.17
fertilised	±0.07	±0.01	±0.03	±0.01	±0.004	±0.003	±9.8	±0.3	±0.5	±0.2	±5.3	±0.2	±0.03	±3.8	±30.5	±42.1
Compost	1.15	0.19	1.29	0.13	0.03	0.09	46.24	5.84	11.78	4.77	41.35	5.21	0.38	23.65	144.37	315.28
fertilised	±0.11	±0.01	±0.06	±0.004	±0.004	±0.003	±9.4	±0.3	±1.2	±0.1	±2.8	±0.2	±0.02	±4.0	±8.9	±37.8
ANOVA																
Main effects																

Fertilisation	*	ns	*	ns	ns	*	ns	**	**	**	ns	*	T	T	*	T
(Fe)																
Crop	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	*	ns	ns	ns	ns	ns
protection (CP)																
2-way																
interactions																
Fe x CP	ns	ns	T	ns	ns	ns	ns	T	ns	ns	ns	ns	ns	ns	ns	ns

Online Resource 5A6A-D. Means and ANOVA data for nutrient analysis of potato terminal leaflets sampled at three growth stages: GS59, GS66, and GS72. Asterix denote p-levels: *=p<0.05, **=p<0.01, ***=p<.001; ns = non significant; T=p=0.049-0.1

Table 5A5A6A	N (%)			P (%)			K (%)			S (%)		
Factor	GS59	GS66	GS72	GS59	GS66	GS72	GS59	GS66	GS72	GS59	GS66	GS72
Means (± SE)												
Conventional CP												
Mineral fertilised	5.60	4.60	3.74	0.37	0.26	0.20	1.95	1.38	1.12	0.37	0.29	0.27
	±0.02	±0.17	±0.12	±0.01	±0.008	±0.007	±0.14	±0.16	±0.16	±0.0005	±0.007	±0.008
Compost fertilised	5.05	3.90	3.24	0.34	0.23	0.17	1.83	1.47	1.13	0.37	0.34	0.34
	±0.07	±0.07	±0.08	±0.01	±0.003	±0.007	±0.18	±0.16	±0.10	±0.01	±0.03	±0.03
Organic CP												
Mineral fertilised	5.37	5.06	4.00	0.36	0.30	0.21	2.04	1.45	1.22	0.35	0.30	0.25
	±0.17	±0.08	±0.10	±0.01	±0.003	±0.004	±0.10	±0.05	±0.05	±0.004	±0.003	±0.007
Compost fertilised	5.10	4.02	3.41	0.35	0.25	0.20	1.89	1.52	1.43	0.35	0.31	0.34
	±0.16	±0.07	±0.09	±0.01	±0.004	±0.005	±0.12	±0.15	±0.14	±	±0.02	±0.03
ANOVA												

Main effects												
Fertilisation (FE)	*	****	***	*	****	**	ns	ns	ns	ns	ns	**
Crop protection (CP)	ns	T	T	ns	*	*	ns	ns	T	T	ns	ns
2-way interactions												
FE x CP	ns	T	ns	T	*	ns	ns	ns	ns	ns	ns	ns

Online Resource	Ca (%)			Na (mg kg ⁻¹)			Mg (%)			Fe (mg kg ⁻¹)		
5B6B												
Factor	GS59	GS66	GS72	GS59	GS66	GS72	GS59	GS66	GS72	GS59	GS66	GS72
Means (± SE)												
Conventional CP												
Mineral fertilised	1.39	1.40	1.45	54.48	31.57	38.70	0.81	0.83	0.97	132.45	111.10	83.14
	±0.04	±0.03	±0.04	±2.08	±6.66	±2.53	±0.03	±0.04	±0.05	±6.48	±9.78	±3.18
Compost fertilised	1.34	1.41	1.60	50.25	35.26	40.12	0.78	0.77	0.92	154.98	120.55	80.52
	±0.06	±0.04	±0.10	±6.02	±6.35	±3.70	±0.04	±0.04	±0.05	±13.91	±27.07	±7.5
Organic CP												
Mineral fertilised	1.38	1.55	1.67	66.26	37.11	31.61	0.80	0.91	1.01	137.59	111.70	89.01
	±0.01	±0.05	±0.04	±4.59	±4.93	±2.65	±0.08	±0.03	±0.04	±5.25	±1.74	±3.83
Compost fertilised	1.36	1.31	1.83	69.32	37.23	34.30	0.79	0.68	0.88	138.57	89.86	87.06
	±0.04	±0.07	±0.04	±11.67	±0.49	±2.56	±0.02	±0.06	±0.07	±6.95	±1.85	±1.15
ANOVA												
Main effects												

Fertilisation (FE)	ns	**	*	ns	ns	ns	ns	ns	**	ns	ns	ns
Crop protection (CP)	ns	ns	*	T	ns	*	ns	**	ns	ns	ns	ns
2-way interactions												
FE x CP	ns	**	ns	ns	ns	ns	ns	*	ns	ns	ns	ns

Online Resource	Cu (mg kg ⁻¹)			Zn (mg kg ⁻¹)			Mn (mg kg ⁻¹)			Mo (mg kg ⁻¹)		
5C6C												
Factor	GS59	GS66	GS72	GS59	GS66	GS72	GS59	GS66	GS72	GS59	GS66	GS72
Means (± SE)												
Conventional CP												
Mineral fertilised	20.11	12.92	9.93	23.94	20.41	27.26	140.86	118.71	186.14	0.50	0.66	0.56
	±1.9	±1.18	±0.68	±0.48	±0.75	±2.18	±10.3	±3.65	±14.57	±0.064	±0.09	±0.07
Compost fertilised	16.63	12.01	9.37	21.47	17.98	26.49	106.57	86.28	167.33	0.67	0.84	0.71
	±0.87	±1.13	±0.53	±0.97	±0.83	±2.71	±15.65	±5.69	±20.48	±0.06	±0.13	±0.11
Organic CP												
Mineral fertilised	915.09	129.68	882.49	22.34	18.69	12.57	47.82	50.36	49.58	0.49	0.62	0.55
	±106.9	±8.36	±46.65	±0.85	±0.27	±0.46	±6.39	±4.68	±8.25	±0.06	±0.06	±0.05
Compost fertilised	959.07	136.26	977.25	21.18	14.91	11.61	30.10	24.41	23.46	0.68	0.76	0.66
	±60.53	±12.1	±80.43	±0.79	±0.56	±0.22	±2.48	±1.18	±0.90	±0.03	±0.02	±0.03
ANOVA												

Main effects												
Fertilisation (FE)	ns	ns	ns	*	***	ns	**	***	ns	**	*	*
Crop protection (CP)	**	***	***	ns	*	**	**	*	**	ns	ns	ns
2-way interactions												
FE x CP	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns

Online Resource	B (mg kg ⁻¹)			Al (mg kg ⁻¹)			Cd (µg kg ⁻¹)			Ni (µg kg ⁻¹)		
SD6D												
Factor	GS59	GS66	GS72	GS59	GS66	GS72	GS59	GS66	GS72	GS59	GS66	GS72
Means (± SE)												
Conventional CP												
Mineral fertilised	21.41	18.82	19.90	48.01	25.30	22.32	1108.72	773.24	629.72	851.41	641.38	510.85
	±0.80	±0.41	±0.407	±8.60	±2.43	±2.71	±122.59	±109.59	±93.18	±77.2	±73.2	±38.7
Compost fertilised	22.03	25.71	26.23	83.28	36.37	23.77	545.65	397.53	318.27	634.37	542.28	444.25
	±0.67	±1.92	±1.46	±15.44	±6.82	±2.47	±50.61	±44.58	±52.73	±54.2	±37.8	±32.8
Organic CP												
Mineral fertilised	22.24	19.75	23.09	59.49	25.07	26.32	1023.61	853.66	609.88	870.1	819.17	628.99
	±0.66	±0.44	±0.775	±8.33	±1.36	±1.74	±160.46	±86.38	±82.057	±86.5	±55.8	±53.1
Compost fertilised	22.02	25.37	31.40	64.81	28.32	35.20	640.89	350.34	331.55	667.94	506.35	534.59
	±0.39	±0.84	±0.85	±5.21	±3.78	±2.35	±103.11	±23.36	±14.35	±56.1	±31.8	±50.4

ANOVA**Main effects**

Fertilisation (FE)	ns	***	***	**	ns	*	*	****	**	ns	**	ns
Crop protection (CP)	ns	ns	*	ns	ns	ns	ns	ns	ns	ns	ns	ns

2-way interactions

FE x CP	ns	ns	ns	*	ns	T	ns	ns	ns	ns	T	ns
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Online Resource 67: Additional data on protein identification search on Tremble, Swissprot, Uniprot and NCBI databases.

Protein spot ID	Accession number	Similar protein name	Matched species	Number of matching peptides	Number of searched peaks	Sequence coverage (%)	MOWSE Score	Expect
49	PP183_ARATH	Pentatricopeptide repeat-containing protein At2g34370, mitochondrial	<i>Arabidopsis thaliana</i>	21	218	39%	65	0.0095
85	C5MR70	Chloroplast manganese stabilizing protein-II (Fragment)	<i>Solanum tuberosum</i>	11	64	41	104	9.1e-08
92	gi 75140959	Protein Thylacoid formation1	<i>Solanum tuberosum</i>			4	49	
153	gi 113379 P14674.1	Alcohol dehydrogenase 2	<i>Solanum tuberosum</i>	12	169	26	47	0.049
172	gi 18140075 AAL	Putative Kunitz-type tuber invertase	<i>Solanum</i>	9	146	37	53	0.01

	60247.1	inhibitor (Fragment)	<i>tuberosum</i>					
211	gi 37718541 CAE 52322.1	1,3-beta-D-glucan glucanohydrolase (Glucan endo-1,3-beta-glucosidase a)	<i>Solanum</i> <i>tuberosum</i>	14	169	44	67	0.00049
238	DGDG2_ARAT H	Digalactosyldiacylglycerol synthase 2, chloroplastic	<i>Arabidopsis</i> <i>thaliana</i>	12	130	29	64	0.012
239	gi 113379 P14674 .1	Alcohol dehydrogenase 2	<i>Solanum</i> <i>tuberosum</i>	12	129	30	52	0.016
269	gi 132123 P26576 .1	Ribulose biphosphate carboxylase small chain 2B, chloroplastic	<i>Solanum</i> <i>tuberosum</i>	10	112	53	78	4.1e-05
407	YCF2_CUSRE	Protein ycf2	<i>Cuscuta</i> <i>reflexa</i>	37	156	18	58	0.048
432	PSBO_SPIOL	Oxygen-evolving enhancer protein 1, chloroplastic	<i>Spinacia</i> <i>oleracea</i>	11	100	40	67	0.0063
552	B0FPD8	Fructose-bisphosphate aldolase	<i>Solanum</i> <i>tuberosum</i>	18	180	49	84	8.3e-06
14	gi 66475036 AA	Dehydroascorbate reductase	<i>Solanum</i>	10	105	51	69	0.00028

	Y47048.1		<i>lycopersicu m</i>					
41	gi 108773138 YP _635647.1	Ribulose biphosphate carboxylase large chain	<i>Solanum tuberosum</i>	19	66	36	133	1.1e10
53	gi 76160951 ABA 40439.1	Dehydroascorbate reductase	<i>Solanum tuberosum</i>	8	62	43	90	2.2e-06
57	gi 108773137 YP _635646.1	ATP synthase subunit beta, chloroplastic	<i>Solanum tuberosum</i>	38	181	67	258	3.6e-23
71	gi 255571379	Photosystem I reaction center subunit N, chloroplast	<i>Ricinus communis</i>			28	379	
75	PSBO_SOLTU	Oxygen-evolving enhancer protein 1, chloroplastic	<i>Solanum tuberosum</i>	14			122	1.8e-08
81	gi 124126	Chymotrypsin inhibitor I, A, B and C subunits	<i>Solanum tuberosum</i>	16			75	
90	gi 12644171 P233	Oxygen-evolving enhancer protein 1,	<i>Solanum</i>	7	78	36	46	0.050

	22.2	chloroplastic	<i>lycopersicu m</i>					
98	gi 255571379	Photosystem I reaction center subunit N, chloroplast	<i>Ricinus communis</i>			28	381	
103	gi 57339044 AAR 14052.2	Catalase (Fragment)	<i>Solanum tuberosum</i>	22	83	47	169	2.9e14
123	PP237_ARATH	Pentatricopeptide repeat-containing protein At3g16610	<i>Arabidopsis thaliana</i>	17	107	21	64	0.011
130	gi 162946521 AB Y21246.1	Catalase	<i>Solanum tuberosum</i>	10	77	17	58	0.0034
146	gi 57339044 AAR 14052.2	Catalase	<i>Solanum tuberosum</i>	12	54	24	84	8.9e-06
150	PSBP_SOLTU	Oxygen-evolving enhancer protein 2, chloroplastic	<i>Solanum tuberosum</i>	10	176	52	74	0.0012
156	GLYM_SOLTU	Serine hydroxymethyltransferase, mitochondrial	<i>Solanum tuberosum</i>	23	143	37	96	7.4e-06

171	CATA2_SOLTU	Catalase isozyme 2	<i>Solanum tuberosum</i>	31			209	3.6e-17
207	gi 799015	putative glycine rich RNA binding protein	<i>Solanum tuberosum</i>	32			346	
209	gi 254798560	glutathione S-transferase	<i>Arachis hypogaea</i>			5	90	
226	gi 1707998 P5043 3.1	Mixture Serine hydroxymethyltransferase, mitochondrial	<i>Solanum tuberosum</i>	31	150	55	150	2.3e-12
	gi 57339044 AAR 14052.2	Catalase (Fragment)	<i>Solanum tuberosum</i>	21	150	45		2.9e-10
236	gi 76160951 ABA 40439.1	Dehydroascorbate reductase-like protein	<i>Solanum tuberosum</i>	12	174	61	66	0.00053
292	gi 108773138 YP _635647.1	Ribulose biphosphate carboxylase large chain	<i>Solanum tuberosum</i>	21	79	29	94	9.3e-07
349	RBL_SOLTU	Ribulose biphosphate carboxylase large	<i>Solanum</i>	18	104	31	92	1.5e-06

		chain	<i>tuberosum</i>					
379	Q52Z99_DUNSA	6-4 photolyase	<i>Dunaliella salina</i>	16	51	27	100	5e-05
386	gi 108773138 YP_635647.1	Ribulose biphosphate carboxylase large chain	<i>Solanum tuberosum</i>			14	66	0.00063
409	gi 108773138 YP_635647.1	Ribulose biphosphate carboxylase large chain	<i>Solanum tuberosum</i>	21	118	21	109	2.9e08
449	CATA_SOLAP	Catalase	<i>Soldanella alpina</i>	16	147	31	63	0.016
499	RCA_SOLPN	Ribulose biphosphate carboxylase/oxygenase activase, chloroplastic	<i>Solanum pennellii</i>	18	159	38	81	0.00021
535	C86B1_ARATH	Cytochrome P450 86B1	<i>Arabidopsis thaliana</i>	21	163	36	63	0.013
584	B0FPD8	Fructose-biphosphate aldolase	<i>Solanum tuberosum</i>	11	108	34	72	0.00016