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# Genotypic variation in the ability of landraces and commercial cereal varieties to avoid manganese deficiency in soils with limited manganese availability: is there a role for root-exuded phytases?

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The marginal agricultural-systems of the Machair in the Western Isles of Scotland often have limited micronutrient availability because of alkaline soils. Traditional landraces of oats, barley and rye are thought to be better adapted to cope with the limited manganese (Mn) availability of these soils. When commercial cultivars are grown on the Machair, limited Mn-availability reduces crop yield and quality. We hypothesised that traditional cereal landraces selected on the Machair acquire Mn more effectively and that this could be linked to exudation of phytase from roots which would release Mn complexed with inositol phosphates. Growth and Mn-acquisition of five landraces and three commercial cultivars of barley and oats were determined in Machair soil. In addition, root phytase activities were assayed under Mn-starvation and sufficiency in hydroponics. In Machair soil, landraces had greater capacity for acquiring Mn and a greater ability to achieve maximum yield compared to the commercial cultivars. Under Mn-starvation, root phytase exudation was upregulated in all plants, suggesting that this trait might allow cereals to acquire more Mn when Mn-availability is limited. In the landraces, exuded phytase activity related positively to relative Mn-accumulation, whereas in the commercial cultivars this relationship was negative, suggesting that this trait may be secondary to an efficiency trait that has been lost from commercial germplasm by breeding. This research shows that cereal landraces possess traits that could be useful for improving the Mn-acquisition of commercial varieties. Exploiting the genetic diversity of landraces could improve the sustainability of agriculture on marginal calcareous lands globally.

Abbreviations – AM, arbuscular mycorrhizal; DW, dry weight; FW, fresh weight; GS, growth stage; IHP, inositol hexaphosphate; ICP-MS, inductively-coupled plasma mass-spectrometry; Kat, katal; NIST, National Institute of Standards and Technology; *p*NPP, *para*-nitrophenol phosphate; PVA, polyvinyl alcohol; RFLP, restriction fragment length polymorphism; RuBisCO, ribulose-1,5-bisphosphate carboxylase oxidase; SASA, Science and Advice for Scottish Agriculture; TCA, trichloroacetic acid.

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## Introduction

Limited availability of micronutrients (Fe, Zn, Mn, Cu, B and Mo) is a common problem in agriculture (White et al. 2012). It has been exacerbated by the success of the green revolution, which has led to increased demand for micronutrients by fast-growing, nutrient-demanding and high-yielding varieties of cereals (Alloway 2008). Limited availability of micronutrients influences productivity in element specific ways (White 2012, White and Greenwood 2013). Manganese (Mn) deficiency in cereals, and some legumes, grown on alkaline soils is a global problem (Marcar and Graham 1987, Holloway et al. 2001, Yang et al. 2007). Regions affected include large areas of southern Australia, Anatolia, Texas, regions of China and the United Kingdom (Reuter et al. 1973, Graham and Rovira 1984, Pallotta et al. 2000, Heitholt et al. 2002, Scholten 2010). On such calcareous soils, Mn-deficiency is chronic and severe, reducing yields by 25–60% compared to crops with adequate Mn-nutrition (Reuter et al. 1973, Graham and Rovira 1984). On these soils Mn exists in a complex range of forms (Marschner 1988) being present in six oxidation states of varying availability to plants (Millaleo et al. 2010). In addition to pH, Mn-availability is strongly influenced by redox conditions and supply is limited in sandy soils, where total Mn concentrations are small (Porter et al. 2004, White and Greenwood 2013).

In plants, Mn has a key role in photosynthesis through its involvement in the water-splitting system of photosystem II (Husted et al. 2009, Millaleo et al. 2010, Broadley et al. 2012, White 2012, White and Greenwood 2013), which has recently been shown to have utility in diagnosing Mn-deficiency through effects on chlorophyll fluorescence (Schmidt et al. 2013). Mn also plays a role in ATP synthesis (Pfeffer et al. 1986), ribulose-1,5-bisphosphate carboxylase (RuBisCO) reactions involved in carbon fixation (Houtz et al. 1988) and the biosynthesis of fatty acids, acyl lipids and proteins (Ness and Woolhouse 1980). In addition, Mn plays a primary role in the activation of, and as a cofactor to, over 30 other enzyme catalysed reactions in plants (Burnell 1988). Manganese is therefore critical to many metabolic processes such as respiration, photosynthesis, synthesis of amino acids and hormone activation (see reviews by Broadley et al. 2012, White 2012 and White and Greenwood 2013 for more detail). Plants that are Mn-deficient appear chlorotic and are more susceptible to diseases, such as take-all (Wilhelm et al. 1988, Broadley et al. 2012, White 2012). Manganese deficiency is traditionally thought to be difficult to overcome by the application of Mn-fertilisers to soil as the added Mn is rapidly converted to unavailable forms

(Reuter et al. 1973). However, recent studies suggest that the application of soluble Mn-fertilisers to the soil is often an effective way to correct Mn-deficiencies, but only if soil pH is also addressed (White and Greenwood 2013). Foliar applications of manganous sulphate are more effective (White and Greenwood 2013), but this is expensive and often impractical for farmers in marginal lands.

The availability of Mn is also influenced greatly by plants and there is large genotypic variation in Mn-acquisition within plant species, with Mn-efficient cultivars able to reach their yield potential on soils with limited Mn phytoavailability without Mn supplementation (Graham 1988). This is, however, not the case where Mn-deficiency is caused by a low inherent Mn content in the soil. The exact physiological basis of the ability to acquire adequate Mn in cereals is unclear, but could include release of organic acid anions (Neumann and Römheld 2001, Rengel and Marschner 2005, Husted et al. 2005), expression of high affinity Mn transporters (Pedas et al. 2005, 2008), positive interactions with soil microorganisms such as Mn oxidising and reducing bacteria (Rengel 1997) or arbuscular mycorrhizal (AM) fungi (Clark and Zeto 1996), increased transpiration, such that Mn mass flow to the root surface is improved (Hebborn et al. 2009, White and Greenwood 2013) and more extensive root systems with more root hairs (Yang et al. 2008, White and Greenwood 2013). Manganese efficiency is apparently not associated with a greater internal economy of Mn (Graham and Rovira 1984, Graham et al. 1985), although a variety of transporters exist for efficient Mn transport across the plasma membrane and tonoplast of plant cells (Lanquar et al. 2010, White 2012). There is also a large genotype by environment interaction in Mn-acquisition, which is often influenced by temperature, soil water status (Pallotta et al. 2000) and interactions with other mineral elements (Husted et al. 2005). Manganese efficiency is often not expressed in solution culture (Huang et al. 1994) suggesting that it is derived from interactions with the soil that increase Mn-availability rather than the uptake of available Mn (Sayyari-Zahan et al. 2009), questioning the role of high affinity Mn-transporters in this process.

Importantly for this study, genotypic variation exists within barley for Mn-acquisition efficiency (Graham 1988, Hebborn et al. 2005), and initial genetic studies have suggested that it might be controlled by a single gene or locus (McCarthy et al. 1988) and, thus, might be transferred easily to elite breeding lines. In fact, more recently, Pallotta et al. (2000) have identified several RFLPs (grouped distally on chromosome 4HS) linked to a locus for Mn-efficiency in barley termed *Mel1*. The role

of this locus in Mn-efficiency in barley has been verified in glasshouse and field studies and it has been adopted in breeding programmes in South Australia (Pallotta et al. 2000).

It has also been reported that organic amendments affect Mn-dynamics in soil both by being a source of Mn and a source of binding sites for Mn. Organic matter with its high cation exchange capacity affects Mn-availability in soils by forming complexes with Mn (Bradl 2004). The possibility that inositol phosphates complex Mn has been suggested (Rodrigues-Filho et al. 2005). Inositol phosphates are generally the dominant form of organic P in soils (George et al. 2006, Stutter et al. 2012) and have the potential to complex much Mn. The stability of these complexes is likely to be a function of pH, with a general rule that as pH increases, stability increases (Martin and Evans 1987). Rhizosphere phytases catalyse the hydrolysis of soil inositol phosphates, thereby releasing inorganic phosphate for uptake by roots (George et al. 2004, 2005), along with any complexed cation. Coordination sites for Mn within the inositol phosphate Mn complex are not known, but have been suggested (Rodrigues-Filho et al. 2005) leading to the possibility that the exudation of phytase by plants may facilitate the release of Mn and other chelated cations.

The Machair, a critical marginal land of great conservation and social importance, resides on alkaline soils with low Mn-availability. The Machair is a fixed dune rotational agricultural system, consisting of calcareous sand found predominantly in the Western Isles of Scotland and northwest Ireland. It is traditionally used for crofting, a low-input/low-output subsistence agricultural system. Limited physical disturbance during cultivation and close proximity between ploughing and seeding allows the natural vegetation to persist, and for part of the year sheep and cattle are grazed on the rotational areas of the Machair. This combination of agricultural practices creates a complex and diverse mosaic of habitats and plant communities (Crawford 1990, 1997, Owen et al. 2001) which sustain a large diversity of invertebrates and birds (UK Biodiversity Group, 1999). Traditionally, the soil is amended with composted seaweed, kelp (*Laminaria digitata* [Huds.] Lamouroux), collected from local beaches (Angus 2001). The Machair is important for the sustained cultivation of rare landraces of oats and barley. Traditionally cultivated cereals include Uist small oats (*Avena strigosa* Schreb.), Uist bere barley (*Hordeum vulgare*) and landraces of rye (*Secale cereale*), either as landraces or commercial varieties, grown primarily for cattle fodder. There are several key differences between commercial cultivars and landraces. Whereas commercial cultivars have been

bred for yield under optimal conditions in mineral soils and for pathogen resistance, landraces have adapted over many generations to very specific environments. In the Western Isles of Scotland, both oats and barley landraces must function well in mixed stands, with no herbicides, variable water availability, strong winds and a large degree of plant competition (Scholten 2010). It is not proven, but it is likely, that one of these landrace adaptations is to low Mn-availability in calcareous soils.

It is important to understand the mechanisms by which landraces cope with limited Mn-availability in order to identify traits and genes that could be deployed in commercial cultivars to improve sustainability in the reduced input agricultural systems of the future. The aim of the study was to determine whether variation exists between landrace and commercial cereal varieties in their ability to grow on soils of the Machair and, if it does, whether this relates to the exudation of phytase from roots. It was hypothesised that landraces of the Machair would secrete more phytase into the rhizosphere, thereby hydrolysing more inositol hexaphosphate and releasing Mn available for uptake by roots.

## Materials and methods

### Cereal responses to soils with altered Mn-availability

#### Mesocosm preparation and plant growth

In April 2011, approximately 400 kg of Machair soil was collected from an area of approximately 1 km<sup>2</sup> at Drimdsdale (57°18'48''N and 7°23'20''W); see Thorsen et al. (2010a, 2010b) for details of the site and soil characteristics. Briefly, the soil was characterised as a non-rocky, brown calcareous or calcareous regosol (Soil Survey of Scotland, 1982). The field was located approximately 200 m from the western shore of the island and sown with bere barley (*H. vulgare*) for silage using a complex farm saved seed mix into a shallow ploughed seed bed reducing the loss of perennial plants. This resulted in a complex plant community dominated by *H. vulgare*, *Lolium perenne*, *Trifolium repens*, *Dactylis glomerata*, *Phleum pratense*, *Festuca rubra* subspecies *arenaria* and *Poa pratensis*. The soil contained 0% clay, 1.5–4.0% silt and 95.5–98.5% sand, with a CaCO<sub>3</sub> content of 60% of total dry weight (DW), pH (measured in H<sub>2</sub>O) in the range of 7.5–8.0, organic carbon content of 1.5% (DW) and water content at field capacity of 16% of dry soil. Soil was sieved using a 10 mm mesh to remove any existing plant material and homogenised in batches of 40 kg using a cement mixer at 75 rpm for 30 min. MnCl<sub>2</sub>·4H<sub>2</sub>O was added to supplement existing

soil Mn content at 0, 1, 2.5, 5, or 10 mg Mn kg<sup>-1</sup> soil to separate batches during homogenization, it is acknowledged that this form of Mn is not usually used for fertilization purposes and is likely to be poorly available upon addition to soil.

A subsample of the remaining unamended soil was used to derive water content at field capacity. Three pots containing untreated soil were weighed then watered to saturation and excess water allowed to drain for 72 h in the glasshouse. In parallel, three pots containing untreated soil were weighed and oven dried. The difference in final weights of the two sets of soils was defined as field capacity.

### **Seed selection, germination, planting and maintenance**

Two Uist bere barley landraces, two common commercial mainland barley cultivars, Optic and Westminster, two Uist small oat (*Avena strigosa* Schreb.) landraces and one commercial mainland common oat (*Avena sativa*) cultivar, one Murkle oat (*Avena sativa*) landrace, Firth, were selected because they were known to differ in ability to grow on limited soil Mn (Scholten, 2010). Seed was sourced from Science and Advice for Scottish Agriculture (SASA), the James Hutton Institute and Maria Scholten (Scottish Rural College, Edinburgh). One hundred seeds of each genotype were germinated for 3 days on agar gel (1% w/v water agar) prior to planting and, when possible, seedlings were planted at the same germination stage (radicles approximately 5 mm in length). Two germinated seeds were sown into each pot which contained 950 g (oven-dried equivalent) of either unamended or Mn-amended soil. Plants were thinned to a single plant per pot after 10 days. Plants were grown in a glasshouse where day length was maintained at approximately 17 h with supplemental lighting allowing maintenance of day length and light intensity at a minimum 200  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . Temperature was allowed to fluctuate between 25 and 15°C. The soils were maintained at approximately 80% field capacity during the growth period by daily watering to weight with distilled water. All mineral nutrients, except Mn, were provided by weekly addition of 25 ml pot<sup>-1</sup> of a standard nutrient solution (3 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 1 mM MgSO<sub>4</sub>, 10 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 80  $\mu\text{M}$  FeEDTA, plus micronutrients [30 nM H<sub>3</sub>BO<sub>3</sub>, 6  $\mu\text{M}$  CuSO<sub>4</sub>, 0.6  $\mu\text{M}$  ZnSO<sub>4</sub>, 42 nM (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>, 12  $\mu\text{M}$  Co<sub>4</sub>(NO<sub>3</sub>)<sub>2</sub>]).

Plants were grown in a randomised replicated block design with each block containing a single replicate of every combination of eight genotypes and five Mn treatments (five blocks of 40 plants in total). Blocks were rotated within the glasshouse every few days to minimise the effect of any potential environmental gradients

within the glasshouse. Emerging weeds were removed throughout the experiment. After 9 weeks growth, and 2 days prior to harvest, the growth stage (GS) of the main stem was scored based on the standard GS scale (HGCA 2006) and tillers were counted. At harvest, visual symptoms of Mn-deficiency were recorded for all plants on the second to last fully expanded leaf (diagnostic leaf, data not presented); the diagnostic leaf was placed in an Eppendorf tube and freeze-dried to determine dry mass and taken for determination of Mn-content. Plants were separated into stem, ear and leaf fractions and dry mass determined after drying for 1 week at 70°C; dry mass of the diagnostic leaf was added to derive total shoot DW. Dried samples of the diagnostic leaf (approximately 50 mg DW) were digested in a MARS-Xpress microwave oven (CEM, Birmingham) in 3 ml AristAR<sup>®</sup> concentrated nitric acid (15 M HNO<sub>3</sub>) at 180°C for 20 min. One milliliter of AristAR 30% hydrogen peroxide (9.8 M H<sub>2</sub>O<sub>2</sub>) was then added followed by a further 20 min of digestion to clear the digests (Johnson et al. 2009). Blank digests and tomato leaf standard (1573a; National Institute of Standards and Technology (NIST), Gaithersburg, MD) were used for quality control purposes. Digests were then made up to 50 ml and the Mn concentrations of digested samples were determined by inductively-coupled plasma mass-spectrometry (ICP-MS; Elan DRC-e, Perkin-Elmer, Beaconsfield, Bucks, UK). Ear material was then separated into seed, awns and husk and the seed material weighed to measure yield per plant.

### **Phytase and phosphatase activity in root exudates of hydroponically-grown cereals**

#### **Hydroponic system and collection of exuded phytase**

Extracellular phytase activities were determined for the eight genotypes used previously. Between four and six replicate pre-germinated seeds (as described above) of each genotype were grown for 2 weeks with and without Mn additions in a hydroponic system contained in a growth cabinet (Sanyo SGC170 Fitotron; Sanyo Gallenkamp Plc, Leicester, UK) with a light intensity of approximately 400  $\mu\text{mol quanta m}^{-2} \text{s}^{-1}$ . Day length was set to 16 h and the cabinet maintained temperatures of 20°C during the day and 16°C at night. Only two Firth oat plants germinated in agar gel (1% water), so there were no replicates of this genotype. Plants were grown in eight 7.5 l hydroponic chambers consisting of a water tight box with reticulated aeration and a suspended lid with holes for plants to grow through with individual plants kept in place by neoprene earplugs (LaserLite, Sperian Hearing Protection, San Diego, CA). Up to 16 plants were grown per chamber, depending on

germination success. All of the hydroponics chambers were filled with Mn-free (–Mn) nutrient solution made up from 100× stock solutions [300 mM NH<sub>4</sub>Cl, 400 mM Ca(NO<sub>3</sub>)<sub>2</sub>, 100 mM Fe EDTA, 1 M KH<sub>2</sub>PO<sub>4</sub>, plus micronutrients (23 mM H<sub>3</sub>BO<sub>3</sub>, 6 mM ZnCl<sub>2</sub>, 1.6 mM CuSO<sub>4</sub>, 1 mM CoCl<sub>2</sub>)] which was used to change hydroponics solutions every 48–72 h. To half of these chambers MnCl<sub>2</sub> was added to achieve a concentration of 6 μM (+Mn). For the first 2 days after transfer to hydroponics, seedlings were provided with a quarter-strength nutrient solution, and for the next 2 days they were provided with half-strength nutrient solution. For the remainder of the experiment plants were supplied full strength nutrient solutions as described above. This nutrient solution is based on Hoagland and Arnon (1950) and provides balanced nutrition to the plants at a constant intensity. Whereas the +Mn treatment will give plants equivalent nutrition to that provided in the optimal growth treatment in the soil experiment, the –Mn treatment will be much more severe than any of the soil treatments, equating to complete Mn-starvation. At the time of each change of nutrient solution, plant positions were swapped to minimise the effects of any potential environmental gradients in the cabinet. Plants were removed from the hydroponic chambers after 2 weeks growth and transferred individually to 100 ml (52 mm internal diameter) transparent culture pots (Gosselin UK, Blackburn, UK) containing 50 ml deionised water. These pots were returned to the growth cabinet and left for 24 h under the same atmospheric growth conditions as previously described to collect exudates. Following the collection of root exudates, roots and shoots were separated and dried in an oven at 70°C for 1 week before determination of DWs of separated tissues.

### Determination of enzyme activities

Phosphomonoesterase (phosphatase) and phytase activities were determined using *para*-nitrophenyl phosphate (pNPP) and myo-inositol hexakisphosphate (IHP) as their respective substrates, as described by George et al. (2004). Briefly, ten times concentrated stocks of both substrates were made up in buffer (150 mM MES, 10 mM EDTA, pH 5.5) with substrate concentrations of 100 mM for pNPP and 20 mM for IHP prior to this experiment. Aliquots of root exudates were made up to 300 μl with buffer (final concentration 15 mM MES, 1 mM EDTA, pH 5.5) with final substrate concentrations of 10 mM for pNPP or 2 mM IHP and incubated at 37°C for 60 min (Richardson et al. 2000). Reactions were terminated with equal volumes of either 0.25 M NaOH (phosphomonoesterase) or 10% TCA (trichloroacetic acid) (phytase),

respectively, at either time zero or at the end of incubation. Concentrations of pNPP or phosphate released (determined by reaction with malachite green; Irving and McLaughlin 1990) during the assay were measured spectrophotometrically relative to standard solutions at 414 and 620 nm, respectively. Enzyme activities were calculated from the difference in concentration over the assay period and expressed as katal per mg root fresh weight and referred to as pnpase and IHPase for activity against pNPP and IHP, respectively.

### Data calculation and statistical analyses

Mn-accumulation (μg Mn shoot<sup>-1</sup>) was calculated as the product of shoot Mn concentration (mg Mn g<sup>-1</sup> DW) and total shoot biomass (g). Relative (%) growth, seed yield, tissue Mn concentration and Mn accumulation per plant was calculated from the mean of each parameter for a plant grown with no added Mn and for each genotype at the soil addition where maximum growth was achieved.

To test for significant effects of Mn treatment, genotype and their interaction, the parametric (ANOVA) and non-parametric (Kruskal–Wallis) analysis of variance tests were applied to response variables. Treatment means were compared by Bonferonni's method ( $P = 0.05$ ) (Aho 2011). All statistical procedures were implemented in either R (R Development Core Team, 2012) and GENSTAT (13th Edn, Rothamsted Research). Response variables were assessed for independence, normality (Shapiro–Wilk statistic), kurtosis and skewness values and homogeneity of variance (Levene's test).

Heteroscedasticity was detected in data for Mn-accumulation, Mn-concentration, phytase activity and DW, particularly for Uist small oat B, this could not be minimised by power or log transformation of the data. Thus, the extended Kruskal–Wallis method was applied to these data and to tiller number and growth stage data; the extended Kruskal–Wallis test implemented in R removes any potential replicate block effects prior to testing for variance between response means (Hothorn et al. 2008).

## Results

### Cereal responses to soils with altered Mn availability

#### Cereal growth stage and development

The commercial cultivars Optic (barley) and Firth (oat) developed more slowly than traditional landrace varieties in soil with smaller Mn-availabilities (<2.5 mg kg<sup>-1</sup>) being significantly ( $P < 0.05$ ) retarded in development

(GS: 60–70; flowering to milk development) compared to that (GS: 80–90; dough development) achieved by landraces after 9 weeks growth (Fig. 1). Significant variation in growth stage was not evident amongst the landraces or the barley cultivar Westminster (Fig. 1). The addition of Mn to soil did not influence number of tillers per plant (Table 1).

### **Cereal productivity**

Commercial cultivars showed a significant increase in shoot biomass ( $P < 0.05$ ) in response to the addition of Mn to soil (Table 1). The soil Mn addition required for maximal productivity of barley cultivar Optic was  $2.5 \text{ mg Mn kg}^{-1}$  ( $P < 0.01$ ). Similarly barley cultivar Westminster and oat cultivar Firth also showed significant differences ( $P < 0.05$ ) between shoot DW at zero Mn addition and peak productivity at  $2.5 \text{ mg Mn kg}^{-1}$ . Additions of Mn greater than  $2.5 \text{ mg Mn kg}^{-1}$  generally caused a decrease in shoot DW in all commercial cultivars. Biomass of the landrace varieties of barley and oats was unaffected by the addition of Mn to soil. Landraces of both barley and oat achieved shoot biomasses under limited Mn-availability between 82 and 100% of those under Mn sufficient conditions. In contrast, commercial cultivars achieved shoot biomasses of 58–62% of those under Mn-sufficient conditions (Table 2). When supplied with sufficient Mn, both commercial cultivars and landraces achieved the same ( $P > 0.05$ ) shoot biomass production.

Similarly, seed yield of landraces did not vary in response to Mn addition to soil. Under conditions of no added Mn, barley landraces were 10–20% more productive than their commercial counterparts, producing up to 3 g more seed dry matter per plant. Landraces of oats were three-fold more productive than the commercial cultivar Firth oat, producing between 7 and 8 g more dry matter and 2.5 g grain yield per plant. Relative yield of the landraces of both barley and oats was 74–100% of the yield in Mn-sufficient conditions, with most varieties achieving greater than 95% of Mn-sufficient yield (Table 2). In contrast, the commercial varieties grown under limited Mn-availability achieved 34–43% of the Mn-sufficient yield.

### **Mn concentration and accumulation**

Mn concentrations in shoot tissue of landraces of barley and oats were unaffected by increasing soil Mn addition, but were significantly greater than the commercial varieties of both species achieving almost double the Mn concentrations. Barley landraces generally exhibited greater ( $P < 0.05$ ) shoot Mn concentrations

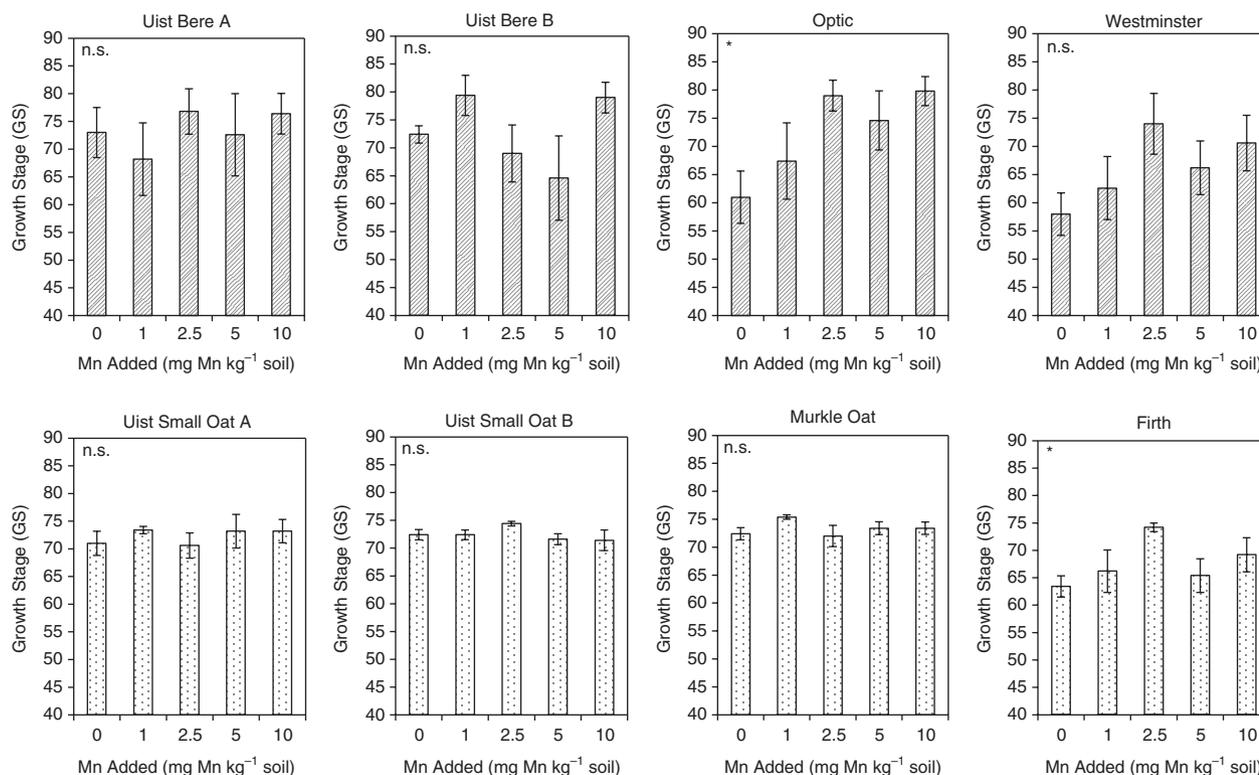
( $22\text{--}37 \mu\text{g g}^{-1}$ ) than oat landraces ( $15\text{--}27 \mu\text{g g}^{-1}$ ). Shoot Mn concentrations responded significantly ( $P < 0.05$ ) to soil Mn additions in commercial cultivars of barley and oat, and shoot Mn concentration in barley was maximal at soil additions of  $2.5 \text{ mg Mn kg}^{-1}$  soil (Table 1). Maximum shoot Mn concentration in Optic and Westminster barley paralleled maximum productivity. Maximal shoot Mn concentrations of  $13\text{--}16 \mu\text{g g}^{-1}$  were achieved at soil Mn additions above  $2.5 \text{ mg kg}^{-1}$ . Firth oat Mn tissue concentration also showed response ( $P < 0.1$ ) to increasing soil Mn content, with maximum shoot Mn concentrations of  $10\text{--}12 \mu\text{g g}^{-1}$  being achieved at soil Mn additions above  $2.5 \text{ mg kg}^{-1}$ .

Landraces of both oat and barley accumulated significantly ( $P < 0.05$ ) more Mn per plant than the corresponding commercial cultivars, such that barley landraces accumulated greater than 4-fold and oat landraces accumulated almost 10-fold more Mn than the commercial varieties. Barley landraces tended to accumulate more Mn per plant than oat landraces (Fig. 2). Shoot Mn-accumulation responded significantly ( $P < 0.05$ ) to soil Mn additions in commercial cultivars of barley, and Mn-concentration in barley was maximal at  $2.5 \text{ mg Mn kg}^{-1}$  soil (Fig. 2). Shoot Mn-accumulation by landraces of barley was unaffected by increasing soil Mn addition, but landraces of oat did show increased Mn accumulation with the largest addition of Mn to soil.

Compared to commercial varieties, landraces showed significantly ( $P < 0.05$ ) greater relative accumulation of Mn under deficient conditions relative to optimum levels of Mn addition (Table 2). Under Mn-deficient conditions, landraces of barley accumulated 85–90% of the Mn accumulated under Mn-sufficient conditions whereas commercial barley varieties accumulated 33–39% of the Mn accumulated under Mn-sufficient conditions. Similarly, under Mn-deficient conditions, oat landraces accumulated 62–100% of the Mn accumulated under Mn-sufficient conditions compared to the 47% achieved by the commercial cultivar Firth.

### **Phytase and phosphatase activity in root exudates of hydroponically-grown cereals**

There was large plant-to-plant variation in phytase (IHPase) activities of root exudates collected from Mn-free hydroponic conditions, which limited the significance of treatment and genotype factors. However, as a main effect, phytase activity was significantly greater in plants exposed to Mn-starvation than in those supplied with sufficient Mn for all genotypes (Fig. 3) with an average of 5.8-fold greater phytase activity in exudates under Mn-starvation. Under



**Fig. 1.** Development stage achieved by eight genotypes of barley and oat after 9 weeks growth in calcareous soils from the Scottish Machair supplemented with a range of added Mn (0, 1, 2.5, 5 and 10 mg Mn kg<sup>-1</sup> soil). Growth stages from flowering (GS60–70) through milk development (GS70–80) to dough development (GS80–90) were variously achieved. Values are means (plus one standard error) of five plants and significant differences in main effects ( $LSD P < 0.05$  genotype = 4.7, Mn treatment = 3.7) and interactions ( $LSD P < 0.05 = 10.5$ ) were established using ANOVA; treatment differences within genotypes were determined using the Kruskal–Wallis test and differences are given as no significance (n.s.),  $P < 0.05$  (\*),  $P < 0.01$  (\*\*) and  $P < 0.001$  (\*\*\*).

Mn-starvation, roots of landraces tended to exude more phytase activity (500–1600 nKat g<sup>-1</sup> root DW) than the commercial varieties (0–700 nKat g<sup>-1</sup> root DW); exuded phytase activity in the presence of Mn was similar for landrace and commercial varieties (Fig. 3). Acid phosphatase (pnpase) activity in root exudates was significantly ( $P < 0.05$ ) less than phytase activity and did not vary significantly between Mn treatments or genotypes (Fig. 3).

A significant positive logarithmic correlation was found for landraces between relative Mn-accumulation and upregulation of phytase activity under Mn-starvation conditions (Fig. 4). By contrast, commercial varieties exhibited a significant negative linear correlation between these parameters (Fig. 4).

## Discussion

Our study provides novel evidence for the potential contribution of root traits to the plant's response to limited Mn availability. Compared to commercial

varieties, landrace varieties of barley and oat selected for cropping on Machair soils were significantly better at maintaining plant development and productivity when soil Mn-availability was limited. Furthermore, our findings suggest that the ability to acquire sufficient Mn under reduced Mn-supply could be related to a significant upregulation of root phytase activity under limited Mn-availability, although this was apparent in all genotypes and not limited to the landraces. On the basis of our data, phytase exudation is not the primary trait to improve Mn-acquisition under limited Mn-availability in the landraces. Selection for increased root phytase activity along with the unidentified primary trait could be a target for breeding cereal varieties that can be cropped sustainably on soils with reduced Mn-availability.

There were striking differences between commercial and landrace varieties of barley and oat in the pattern of development, dry matter accumulation and Mn-acquisition in response to soil Mn addition. Commercial varieties of barley and oat exhibited delayed development and reduced productivity in the

**Table 1.** Mean number of tillers, shoot DW (g), shoot Mn concentration ( $\mu\text{g g}^{-1}$  shoot) and seed yield (g) per plant of eight barley and oat genotypes grown in Mn-deficient calcareous soils from the Scottish Machair amended with five Mn treatments (0, 1, 2.5, 5 and 10 mg Mn  $\text{kg}^{-1}$  soil). Values are means of five replicates and significant differences in main effects and interactions were established using ANOVA and least significant difference (LSD  $P < 0.05$ ) are given.

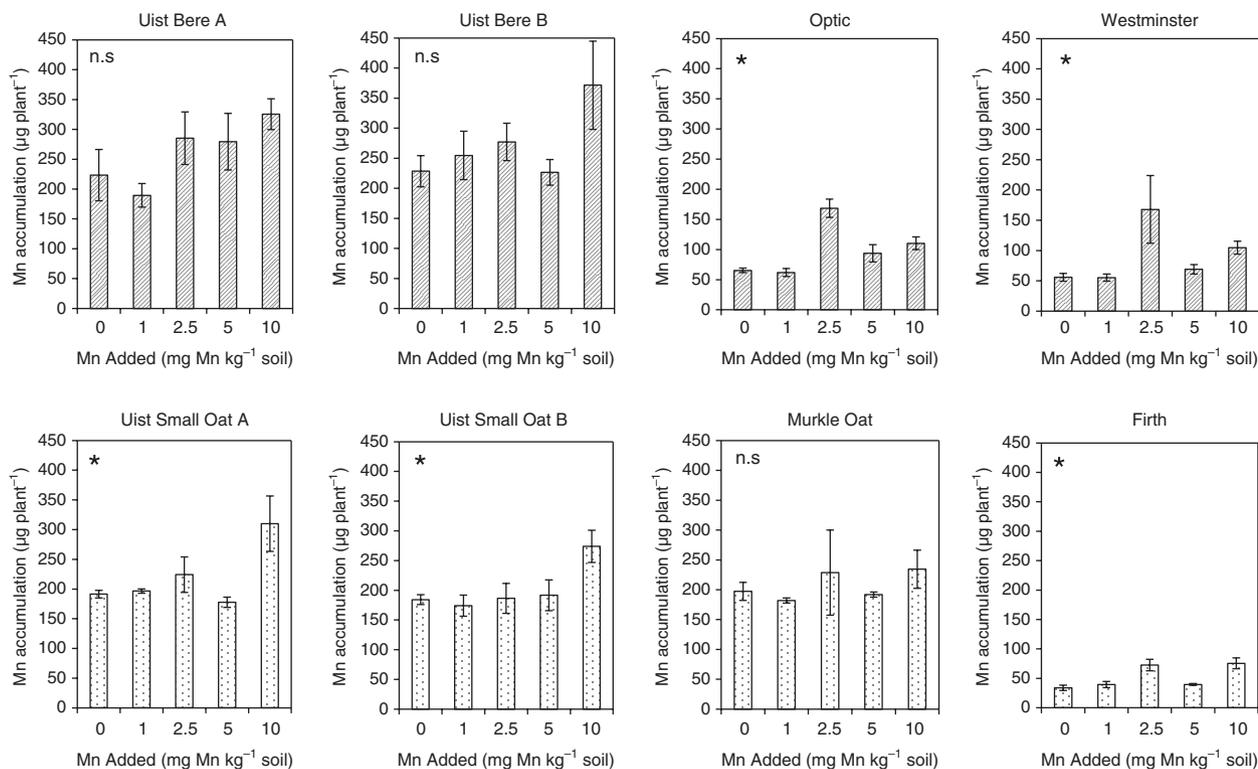
Species/genotype	Mn added (mg Mn $\text{kg}^{-1}$ soil)	No. tillers	Shoot DW (g)	Mn concentration ( $\mu\text{g g}^{-1}$ shoot)	Seed yield (g)			
<i>Barley</i>								
Uist bere A	0	5.8	10.33	21.4	4.6			
	1	5.6	7.68	26.7	3.16			
	2.5	5	10.57	27.2	4.6			
	5	5.6	9.73	28.2	3.76			
	10	5.2	10.53	31	4.62			
Uist bere B	0	3.8	9.21	25.5	3.26			
	1	5.8	11.23	22.8	4.44			
	2.5	4.4	10.17	27.7	3.48			
	5	6	8.66	28.4	3.6			
	10	4.8	9.43	38.2	3.94			
Optic	0	10.4	7.97	8.3	1.66			
	1	10.4	7.84	7.9	1.5			
	2.5	9.6	13.08	13	4.84			
	5	11.8	10.05	9.1	3.1			
	10	9.2	10.03	10.9	3.48			
Westminster	0	9.6	6.71	8.3	1.58			
	1	10.6	7.65	7.27	1.74			
	2.5	10.8	10.8	15.62	3.7			
	5	9.4	7.83	8.85	1.88			
	10	11.8	10.71	9.81	3.5			
<i>Oats</i>								
Uist small oat A	0	8.2	10.81	17.8	3.18			
	1	8.8	11.43	17.3	3.02			
	2.5	7.2	11.41	19.4	2.98			
	5	8	10.77	16.5	3.1			
	10	8.6	11.92	26.9	3.18			
Uist small oat B	0	6.2	11.58	16	3.38			
	1	7.2	10.3	17.5	2.72			
	2.5	7	10.23	18.3	3.02			
	5	8	10.11	18.6	2.66			
	10	6.2	12.02	22.9	3.52			
Murkle oat	0	6.6	12.36	15.9	3.42			
	1	7.6	11.66	15.6	3.36			
	2.5	6.8	11.46	22.3	2.92			
	5	6.8	11.78	16.4	3.14			
	10	8	11.77	20.4	3.56			
Firth	0	4	4.2	8	0.9			
	1	4.2	4.45	9.1	1.12			
	2.5	4.4	7.28	10	2.22			
	5	4.2	4.7	8.5	1.06			
	10	4.2	6.69	11.4	2.16			
	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value	LSD	<i>P</i> -value
Genotype	1	<0.001	0.9	<0.001	3.5	<0.001	0.5	<0.001
Mn treatment	0.8	0.245	0.7	<0.001	2.8	<0.001	0.4	<0.001
Interaction	2.2	0.732	1.9	<0.001	7.8	0.819	1.2	0.001

absence of supplementary soil Mn, but development and productivity were stimulated by the addition of Mn above a threshold level ( $\geq 2.5$  mg Mn  $\text{kg}^{-1}$  soil). This is a common response to deficiency of Mn (Hebburn

et al. 2005) and other micronutrients (e.g. Cu: Alloway 2008). Furthermore, maximum development rate and dry matter production of commercial barley and oat varieties was achieved when tissue Mn-concentrations

**Table 2.** Relative (%) shoot DW (g), seed yield (g), Mn concentration ( $\mu\text{g g}^{-1}$ ) and Mn accumulation of barley and oat genotypes in unamended Machair soil in comparison to soil optimally fertilized with Mn. Values are means of five replicates and standard errors are shown in parenthesis.

Genotype [relative parameter (%)]	Uist bere A	Uist bere B	Optic	Westminster	Uist small oat A	Uist small oat B	Murkle oat	Firth
Shoot DW	97.7 (7.8)	82.0 (9.8)	60.9 (4.4)	62.1 (6.8)	90.7 (5.7)	96.3 (7.0)	100.0 (5.5)	57.7 (4.6)
Seed yield	99.6 (12.6)	73.4 (14.3)	34.3 (7.0)	42.7 (17.8)	100.0 (14.0)	96.0 (7.1)	96.1 (4.6)	40.5 (7.4)
Mn concentration	85.5 (12.1)	111.7 (17.8)	63.6 (6.4)	53.1 (18.9)	65.9 (14.2)	69.9 (7.8)	100.0 (35.8)	79.7 (9.2)
Mn accumulation	84.6 (17.7)	89.7 (16.8)	38.8 (4.0)	33.2 (11.9)	61.8 (10.1)	67.3 (7.3)	100.0 (9.8)	46.7 (8.3)

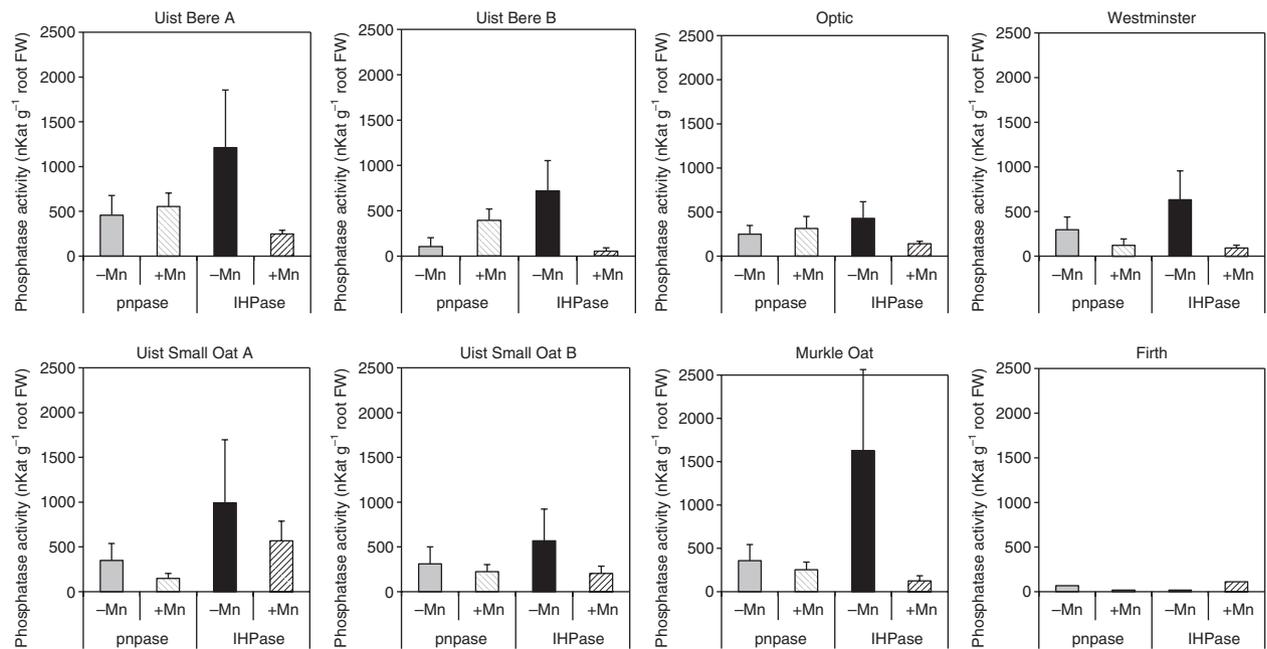


**Fig. 2.** Mn accumulation ( $\mu\text{g plant}^{-1}$ ) by eight barley and oat genotypes after 9 weeks growth in calcareous soils from the Scottish Machair supplemented with a range of added Mn (0, 1, 2.5, 5 and  $10\text{ mg Mn kg}^{-1}$  soil). Values are means (plus one standard error) of five plants and significant differences in main effects ( $\text{LSD } P < 0.05$  genotype = 35.4, Mn treatment = 28.0) and interactions ( $\text{LSD } P < 0.05 = 79.2$ ) were established using ANOVA; treatment differences within genotypes were determined using the Kruskal–Wallis test and differences are given as no significance (n.s.),  $P < 0.05$  (\*),  $P < 0.01$  (\*\*) and  $P < 0.001$  (\*\*\*)

and total Mn-accumulation were greatest, suggesting that increased soil Mn-availability might stimulate plant growth by alleviating tissue deficiencies in Mn. By contrast, the lack of growth response to soil Mn addition by landraces of barley (*H. vulgare*), small oat (*A. strigosa* Schreb.) and murkle oat (*A. sativa*), despite increases in tissue Mn-concentration, suggested that soil Mn availability was not limiting to growth. Interestingly, landraces of barley and oat maintained consistently greater tissue Mn-concentrations (up to c. 2–4-fold higher) than commercial varieties, resulting in larger Mn requirement to achieve maximum productivity, which suggested that landraces were less efficient at

utilising Mn to build tissue dry matter. Taken together, these observations suggest that commercial varieties experienced limiting availability of soil Mn compared to landraces and/or that uptake and storage of Mn was relatively unrestricted in the landraces.

The commercial cultivars assessed here exhibited a reduction in yield under the conditions of limited Mn-availability imposed by the calcareous sands of the Machair, as has been reported previously for barley grown on calcareous sandy soils in Australia (Reuter et al. 1973, Graham et al. 1983) and wheat and soybeans grown in other parts of the world (Marcar and Graham 1987, Heitholt et al. 2002). Yield reductions in

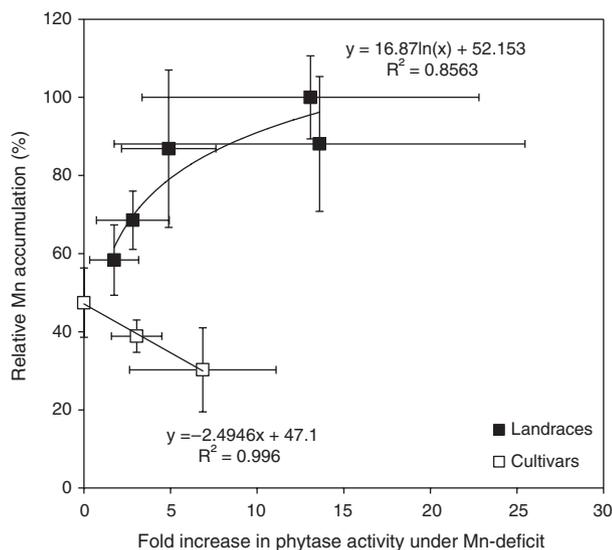


**Fig. 3.** Activity of phosphatases (nKat g<sup>-1</sup> root FW) against the substrates *para*-nitrophenol phosphate (pnpases) and inositol hexaphosphate (IHPases) in root exudates of eight genotypes of barley and oats grown hydroponically with or without added Mn (+Mn and -Mn, respectively). Plants were grown for 2 weeks in hydroponics solutions, which provided all nutrients adequately except Mn, which was either left absent or was supplemented with 6  $\mu$ M Mn as MnCl<sub>2</sub>. Plants were grown in a controlled environment cabinet and solutions were changed regularly. After 2 weeks of plant growth in hydroponics, exudates were collected in deionised water over a 24 h period. Values are means (plus one standard error) of up to six plants and significant differences in main effects for genotype (LSD  $P < 0.05$  pnpase = 255.7 and IHPase = 731.2) and Mn treatment (LSD  $P < 0.05$  pnpase = 127.8, IHPase = 365.6) and interactions (LSD  $P < 0.05$  pnpase = 361.6, IHPase = 1034.0) were established using ANOVA.

the present study were more severe than the 20–25% yield loss observed for barley in Mn-deficient soils in Denmark (Hebborn et al. 2005). Our data confirms the largely anecdotal evidence that traditional landraces of cereals are more productive than commercial cultivars in the calcareous Machair soils of the Scottish Western Isles, probably because of greater Mn-acquisition by the landraces. In the absence of supplementary soil Mn, barley landraces produced 32–60% more biomass and 1.7–2.9-fold more grain than the commercial varieties Optic and Westminster, whereas small oat and murkle oat produced three-fold more shoot biomass than the commercial oat cultivar Firth. Furthermore, maximal grain yield of barley landraces on Machair soil were comparable to those of commercial barley varieties, whereas grain yield in oat landraces exceeded that of the commercial oat variety Firth, suggesting that soil physical and/or microbiological properties might additionally limit productivity of commercial oat varieties in Machair soils (Vink et al. 2014, Thorsen et al. 2010a, 2010b). These data provide evidence that long-term breeding of commercial cultivars of cereals has potentially attenuated or eliminated traits that promote productivity in low-input agricultural systems. By contrast,

seed-saving over many generations to create the Western Isles landraces has incidentally selected traits that are beneficial to cereal production in low-input calcareous Machair soils, including improved Mn-acquisition under conditions of reduced Mn availability.

The plant traits that might underlie differences in the availability, acquisition and efficient utilization of Mn remain speculative. It is noteworthy that genotypic variation in Mn-efficiency in barley reported previously was expressed only when plants were grown in soil and not when grown in solution culture, suggesting that root–soil interactions are key to efficient Mn use (Huang et al. 1994). Candidate root traits expressed by landraces could include root architecture, such as the topsoil foraging root ideotype proposed to optimise acquisition of Mn, and other elements with restricted soil mobility (White et al. 2013). Other candidate root traits that might improve Mn delivery to and uptake by the root include increased transpiration (Hebborn et al. 2009), enhanced root secretion of protons, phytosiderophores and organic acids to release Mn, from calcareous soils (Römheld 1991, Treeby et al. 1989) or interaction with the soil microbial community to increase Mn-availability in the rhizosphere (White et al. 2013).



**Fig. 4.** Relative Mn-accumulation (%) in the shoot ( $n=5$ ) of eight genotypes of barley and oat in relation to the fold-change in root phytase activity ( $n=6$ , with the exception of Murkle Oat, where  $n=1$ ) in Mn-free compared to Mn-supplemented hydroponic solution culture. Data for landraces and commercial cultivars are plotted separately and regression analysis is shown for landraces (logarithmic curve) and commercial cultivars (linear fit). Error bars are one standard error from the mean.

Whereas the extent of genotypic variation in these candidate Mn-efficiency traits remains to be explored, this study has highlighted a potential role for root-exuded phytase enzymes. Although phosphatase with activity against pNPP was present in the root exudates of all landraces and commercial cultivars, this non-specific enzyme activity was generally small and did not respond to changes in Mn supply. By contrast, the activity of phytases that specifically hydrolyse IHP was upregulated (2–14-fold increase, on average) under Mn-deprivation in all plant genotypes. Elevated root phytase activity is likely to enhance the mineralisation of organic-P forms in the soil and release Mn complexed with organic-P compounds (Rodrigues-Filho et al. 2005), thus increasing Mn-availability to the plant. Phytase activity exuded under Mn-deprivation was considerably greater than phosphatase activity (c. two-fold on average) for all genotypes, but was, on average, 50% less than phosphatase activity exuded when Mn was supplied to the roots. Furthermore, upregulation of exuded phytase activity in response to Mn-deprivation tended to be greater in the landraces than in the commercial varieties. To our knowledge this is the first report of the upregulation of exuded phytase activity by plant roots in response to a stimulus that is not related to phosphorus acquisition.

Among the landraces, the fold change in exuded phytase activity under Mn-deprivation correlated positively with the ability to accumulate Mn in Machair soil; by contrast, among the commercial cultivars, there was a negative correlation between these two parameters. This might indicate that phytase exudation works in tandem with another Mn-efficiency trait in the landraces that is absent from the commercial cultivars, such as high affinity Mn-transporters (Pedas et al. 2005, 2008, Cailliatte et al. 2010, Millaleo et al. 2010), rhizosphere microbial communities with Mn redox activities (Rengel 1997) or root hair distributions and root system architectures that optimize exploitation of soil Mn (Yang et al. 2008). Understanding the complex of traits that lead to enhanced landrace productivity and Mn acquisition could be critical to improving the performance of commercial varieties on Mn-poor soils and should become a research priority in this area.

The farming system used in the Machair is unique, being low intensity and reliant on the addition of rotted, washed seaweed and animal manure to fertilise the soil. The large IHP content of some animal manure (Turner and Leytem 2004) and the unknown IHP contents of seaweeds might favour optimal productivity of cereal varieties that exhibit enhanced IHP-specific phytase activity, rather than more general increases in phosphatase activity, to alleviate nutrient deficiencies (P and micronutrient metals). This study shows the value of searching for traits for agricultural sustainability in older cultivars, landraces and wild relatives of cereals, whether to improve cereal productivity in the globally-rare but locally-valuable Mn-poor marginal systems such as the Machair or to identify traits that might improve productivity in other nutrient-poor soil systems worldwide.

## Conclusions

There is sufficient evidence provided by this investigation to suggest that traditional landraces of barley (*H. vulgare*), small oat (*A. strigosa* Schreb.) and common oat (*A. sativa*) have greater Mn-acquisition under limited Mn-availability in Machair soil than their commercial cultivar equivalents. This ability exhibited by traditional landraces appears linked to their greater acquisition of Mn from the soil, which could be associated with a number of Mn-efficiency traits. In addition, landraces of barley appear to require much greater concentrations of Mn in shoot tissue than commercial equivalents to obtain the same level of productivity. However, there appears to be no difference in maximum productivity exhibited by landrace and commercial varieties when both are supplied with sufficient Mn. Exudation of phytase by

all plant lines was upregulated in response to Mn-starvation but could not explain the greater ability of landraces to acquire Mn under limited Mn-availability directly. Phytase exudation could be one of a range of traits possessed by the landraces to enhance their acquisition of Mn. Greater understanding of these traits will increase our ability to sustainably manage reduced Mn-availability agricultural systems on a global scale.

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