COST Action Project

FA 0905 Mineral-Improved Crop Production for Healthy Food and Feed

Final Conference

Agronomic, Molecular Genetics and Human Nutrition Approaches for Improving the Nutritional Quality and Safety of Food Crops

17-19 March 2014, Ela Quality Resort, Antalya-Belek, Turkey

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Session-I:

Soil-Plant Interactions and Physiology

Agronomic Approaches for Increasing the Zinc Concentration in Cereal Grains

E Frossard, SB Aciksöz Özden, F Aghili, T Dürr-Auster, HA Gamper, A Gramlich, R Grüter, J Mayer, P Mäder, R Schulin, S Tandy, C Thonar

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ABSTRACT

Insufficient zinc (Zn) intake by people consuming cereals as staple food is often related to a too low Zn concentration in grains, which in turn is related to limited Zn availability in soils where cereals are grown. The question we are asking here is: which measures could be used at the cropping system level to increase the Zn concentration of cereal grains? Three approaches might be taken: (a) those with the aim to increase the availability of Zn in soil for uptake by roots (including the supply of available Zn from external sources), (b) those addressing the capability of the plants to access soil Zn (through improved root traits and/or arbuscular mycorrhizal fungi (AMF)), and (c) those aiming at enhanced plant Zn translocation into the grains. Approaches of type (b) and (c) may include both genetic (including selection of cultivars) and agronomic approaches aiming at influencing plant physiological activity e.g. by enhancing the N nutrition status, while the approaches of type (a) are essentially agronomic.

The focus of this presentation will be on agronomic approaches to increase Zn concentration in cereal grains. Using results from glasshouse and field experiments we will first show that green manuring, farmyard manure input, and adapting crop rotation could be valuable options in addition to mineral Zn fertilizer applications. Then we will show that, although AMF hyphae can by themselves take up and transport significant amounts of Zn to the plants, Zn uptake is strongly increased when AMF hyphae and roots explore together the same soil volume. Afterwards we will show how N nutrition can improve the uptake and transfer of Zn from the soil to the grain. Finally, we will see whether the implementation of these measures in long-term field experiments really impact soil Zn extractable with DTPA (taken as a proxy for plant available Zn) and the Zn concentration of crops grown on these trials.

The production by roots and microorganisms of organic molecules (organic acids, amino acids, and siderophores) able to complex Zn from soil particles has been studied especially as a response to Zn deficiency. The input of organic matters low in Zn either partly decomposed as farmyard manure, or fresh as green manure, can lead to the release of such compounds and therefore to soil Zn release. Furthermore, the mineralization of organic N added with these organic matters results in the release of protons which will decrease, at least locally, soil pH and increase soil Zn solubility. These hypotheses have been tested in the work of Soltani et al. (2014) and of Aghili (2014). Soltani et al. (2014) showed in a 2-years field experiment that red clover and sunflower were able to increase dissolved organic carbon and amino acids concentrations in the soil solution of a calcareous soil. These changes were paralleled by an increase in Zn content in the grains of wheat growing after these crops. This increase in Zn concentration was however depending on the wheat cultivar: while a Zn efficient cultivar showed higher Zn concentration in grain, this was not the case in a non-efficient wheat cultivar. Aghili (2014) showed that the introduction in the same calcareous soil of sunflower and red clover green manures led to strong increases in soil Zn extractable with DTPA and in soil Zn uptake by wheat. The effects of green manure input on Zn grain concentration were close to those observed with a single addition of $ZnSO_4$. This was explained by the release of complexing substances, of mineral N and of protons following the mineralization of these green manures.

The positive effect of AMF on Zn uptake by crops has been largely described in the literature (Cavagnaro, 2008; Lehmann et al., 2014). The current paradigm is that AMF hyphae allow plants to take up more Zn by enlarging the soil volume that is explored by roots. Aghili (2014) checked this hypothesis using a compartmented system allowing the growth of only roots, of only AMF hyphae or of both AMF hyphae and roots in a nutrient rich compartment and by labelling the Zn source added to this compartment with radioactive Zn. She found that Zn uptake from this compartment was lower when it

was explored by AMF hyphae alone and maximum when both roots and hyphae explored it. This led to the new hypothesis that roots and hyphae would "cooperate" e.g. with roots and rhizosphere microorganisms exuding molecules able to complex Zn which could then be taken up both by roots and AMF hyphae.

An optimum nitrogen nutrition is needed for the production of proteins that are essential for Zn acquisition and uptake (phytosiderophores, transporter proteins), for Zn transport within the plant (transporters, chelators), and for Zn storage in the seed (storage proteins) (Cakmak et al., 2010). Although the role of sulfur has not yet been intensively studied in this respect, we hypothesize that sulfur nutrition might also have a strong impact on Zn content in cereal grains.

The results presented here suggest that cropping systems having a diverse rotation including legumes, in which organic matter is regularly added in the form of farmyard manure or/and green manure, and that maintain a large diversity of AMF and a high soil biological activity, would be able to deliver cereal grains with a higher Zn concentration and would present a higher soil DTPA extractable Zn compared to systems e.g. that would not receive any organic inputs. This was tested in two Swiss field experiments, the DOK field experiment located close to Basel and the ZOFE field experiment located close to Zurich. We observed indeed that the inputs of organic matter adding little amounts of Zn (farmyard manure, plant residues), increased soil Zn extractable with DTPA. The effect of the cropping system on the Zn concentration of wheat grain in the DOK experiment was however limited, probably because of the high initial level of Zn extractable with DTPA in this soil. The same type of field trial should be established on soils with a lower Zn availability to test the effects of such cropping systems on the Zn concentration in cereal grains.

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Options to Minimize Cadmium and Arsenic Contamination in Rice

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INTRODUCTION

Rice is the staple crop for almost half of the population in the world. Unfortunately, rice is also known to contribute substantially to the human intakes of the toxic trace elements cadmium (Cd) and arsenic (As) (Meharg et al., 2009; Meharg et al., 2013). Cadmium has a relatively high mobility in the soil-plant system, whereas the mobility and bioavailability of As increase markedly under the flooded paddy conditions (Zhao et al., 2010). Accumulation of these elements in rice grain may pose a risk to human health well before phytotoxicity occurs. Contamination of these elements is a major concern for rice production in some areas of China. Practical solutions are urgently needed to mitigate the contamination of Cd and As in the food chain.

SOIL MANAGEMENT OPTIONS

During its rapid industrialization over the last three decades, China has not been able to avoid the previous mistakes made by some developed countries. Farmlands are increasingly being contaminated with heavy metals/metalloids and other contaminants. However, soil contamination is not the only reason for the problem of toxic metal contamination in crops. A meta data analysis show that soil acidification is one of the most important reasons causing the widespread exceedence of Cd in rice grain in southern China, where many soils are naturally acidic and have been subjected to further acidification due to long-term uses of nitrogen fertilizers. Soil acidification increases Cd solubility and bioavailability to plants. At pH < 5.0, rice grain can exceed the Cd limit (0.2 mg/kg in China) even when the soil is not contaminated (<0.3 mg Cd/kg soil). Therefore, liming of acidic soils is a practical solution that can substantially decrease Cd accumulation in rice. In the case of As, arsenite is mobilized during the reductive processes occurring in the anaerobic paddy soil, which is subsequently taken up by the silicon/arsenite transporters into rice roots (Ma et al., 2008). Both greenhouse and field trials have shown that aerobic cultivation or alternate wed-dry management is the most effective measure to decrease As bioavailability and accumulation by rice (Li et al., 2009; Stroud et al., 2011; Norton et al., 2012). However, this runs the risk of increasing Cd accumulation in rice grain if the soil is acidic and/or contaminated by Cd because aerobic conditions favour Cd uptake (Meharg and Zhao, 2012). Under flooded conditions, applications of organic manure or straw incorporation can further exacerbate the problem of As accumulation in rice because inputs of organic matter promote anaerobic conditions. Fertilization with silicon can reduce the accumulation of As and Cd, whereas P fertilizers may increase As accumulation in rice.

POTENTIAL OF CROP BREEDING

Genotypic variation in the concentration of Cd and As in rice grain is substantial; variations of >10 fold and 3.5-35 fold have been reported for Cd and As, respectively (Ueno et al., 2009a; Norton et al., 2012). The magnitude of genotypic variation is far greater than that observed for essential nutrients because the latter are more tightly regulated during uptake and translocation. The large genotypic variation presents good opportunities to employ crop breeding for low accumulation cultivars, especially for Cd. For As the genotype x environment interactions can be substantial (Norton et al., 2012). Quantitative Trait Loci (QTLs) have been reported for Cd and As in rice grain (Ueno et al., 2009a; Ueno et al., 2009b; Norton et al., 2010). A number of genes controlling Cd or As uptake and translocation have been identified (Ma et al., 2008; Ueno et al., 2010; Uraguchi et al., 2011; Ishikawa et al., 2012; Sasaki et al., 2012). Alleles associated with low accumulation under different soil environments are particularly useful. Molecular markers can be developed to assist breeding for low accumulation (Ishikawa et al., 2012). Transgenic approach has also been shown to be effective in decreasing Cd accumulation in rice grain (Ueno et al., 2010).

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Selenium Uptake and its Utilization Efficiency of Wheat (*Triticum aestivum* L.) and Oilseed Rape (*Brassica napus* L.)

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INTRODUCTION

Selenium (Se) has been identified as an essential micronutrient for humans, animals and microorganisms because of its role in antioxidant enzyme, glutathione peroxidase (Terry et al., 2000). Deficiency of Se can result in many health disorders such as arthritis, heart disease, hypothyroidism, asthma and weakened immune systems (Whanger, 2004). The recommended daily intake (RDI) of Se is 55 µg per day, but its intake is not sufficient for many people in Europe and Asia (Broadley et al., 2010). In addition, a large proportion of the world's population uses foods with a low level of Se (Combs 2001). Therefore, the demand for agricultural products with a higher Se content has increased. Se has not been considered a necessary nutrient for plants, but plants play important roles in moving Se into the food chain (Broadley et al., 2010).

In Finland Se availability in the soil is low (eg. 0.04 – 0.7 µg kg^{-1}) and that is why Se-enhanced mineral fertilizers have been used in the fields since 1984 to secure the recommended content in plants for feed and food (Seppänen et al., 2010). Plants ability in uptake and translocation of Se is different and they are able to absorb inorganic forms of Se such as selenate ($Se_2O_4^{-2}$) and selenite ($Se_2O_3^{-2}$) or organic form such as selenomethionine (SeMet) (Terry et al., 2000). In this study, wheat and oilseed rape Se utilization efficiency was investigated in field and greenhouse experiments. Moreover, the bioavailability of organic Se form (Se- enriched leaf and straw residues) to plants was compared to inorganic form ($Na_2SeO_4^{-2}$).

METHODS

A 2-years field study (2011-2012) was undertaken to monitor the uptake, translocation and accumulation of Se applied during different growth stages of plants. Three different levels of Se (0, 7.2 and 25 g Se hec^{-1}) were applied. In addition, in 2012, two levels of nitrogen (-N, without fertilizer and +N with 80 kg hec^{-1} N fertilizer) were applied. In greenhouse experiments Brassica napus L. treated with three levels of inorganic Se (0, 7 and 140 µg Se kg^{-1} soil) and organic Se (straw and leaf residues, 0 and 7 µg Se kg^{-1} soil) were added. Plants and soil samples after harvesting were prepared for Se analysis by ICP- mass spectrometry.

RESULTS AND DISCUSSION

In field experiments 2011, the recovery of the added Se was higher in rapeseed (33% and 45%) than in wheat (30% and 29%) under low and high Se fertilization levels, respectively. However, there was no significant difference in SeUE between the two crops. In 2012, the SeUE of both crops was significantly lower probably due to higher precipitation causing lower Se bioavailability in the soil. The soil analyses confirmed this assumption; the residual Se in soils was higher in 2012 compared with that in 2011. Moreover, an interaction between Se and N was observed, at lower Se application level foliar N improved both Se uptake and SeUE.

In greenhouse the Se uptake of oilseed rape plants was 66 and 73% (140 and 7 µg Se kg^{-1} soil, inorganic Se), 26% (7 µg Se kg^{-1} soil, Se- enriched leaf residues) and 4% (7 µg Se kg^{-1} soil, Se- enriched straw residues). Similarly as in field experiments, Se uptake was high but the mobilization to seeds was low. The bioavailability of Se in organic plant residues, especially in straw, is low. The results show that majority of the fertilizer Se in circulated back to soil in straw residue, but due to the low bioavailability, annual Se application is required to ensure adequate content in harvested seed.

CONCLUSIONS

Se uptake of oilseed rape was higher than that of wheat, but the mobilization to seeds was low and Se accumulated in stems and siliques. An interaction between Se and N was observed so that N slighly improved Se uptake. We conclude that the bottleneck in SeUE seemed to be inefficient translocation to seeds.

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Silicon Decrease Cadmium in Crops: A Field Study

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INTRODUCTION

Cadmium is a toxic element, which is known to create health problems (Nordberg 1996). Wheat and potato are crops that accumulate cadmium and when these crops, or products originating from them, are much consumed, the intake of cadmium is high (Hellstrand and Landner 1998). Our recent research shows that the presence of silicon decreases the Cd accumulation in grains of wheat grown in nutrient solution (Greger and Landberg 2008). Silicon decreases the translocation of cadmium from root to shoot and this decrease is most pronounced in wheat cultivars with high accumulation of cadmium in their grains. The aim of the present study was to find out if the same effect could be found in field grown crops of wheat as well as in potato, carrot and onion by adding silicon to the soil.

METHODS

Field experiment set up: In this investigation different silicon additives were tested; 1) liquid potassium silicate from YARA, 2) amorphous SiO_2 Microsilica from Elkem and 3) powder form of Solaritt (mixture of $CaSiO_3$, $Ca_3Si_2O_7$ and CaO with liming effect) from ELKEM. Silicon was applied in four different farmers fields in Hedmark county, Norway, one months after sowing, 4 July 2013. Silicon was applied as 500 kg Si per ha was applied to each parcels being 3 m^2. The soils were of alum shale type, light clay with Cd contents around 2 mg per kg and pH about 6.4. Plants investigated were winter potato *(Solanum tuberosa)*, carrots *(Dacus carota)*, yellow onion *(Allium cepa)* and spring bread wheat *(Triticum aestivum)*. Plants were harvested at maturity 6th of October.

Analyses: The edible plant parts were collected. Samples of similar size of each species were chosen to be able to compare. Four specimens from each parcel were pooled to one sample. The edible parts of the plants were washed in redistilled water and about 1 cm transects in the middle of the carrot, potato and onion was cut out to get 1 g dried sample. In the case of wheat, the grains from 35 - 50 wheat plants were collected and pooled together. Plant materials were dried 24 - 72 hours in 105°C, weighed and wet digested in $HClO_4$:HNO_3 (3:7 v/v). The wet digested material was then analysed on Cd concentration using atomic absorption (SpectrAA 55B, VarianInc) and standard addition to eliminate the matrix effect.

Calculations and statistics: The field investigation was performed in 4 replicates, randomly spread. Statistical calculations were performed with JMP 10.0 (SAS Inst. Inc.) Both ANOVA and *t*-tests (Tukey-Kramer) were utilized to compare treated with non-treated.

RESULTS AND DISCUSSION

All plants investigated contained cadmium (Fig. 1). In all cases, either significantly ($p \leq 0.05$) or as a tendency silicon addition decreased the cadmium accumulation in the edible plant part. This was the case for all three Si additives. This means that the effect was shown not only on a Silicon accumulator (wheat, which is a monocotyledon of gramineous type) but also on not Si accumulators of both monocotyledon (onion) and eudicotyledonous (potato and carrot) type.

One might expect that Si addition increases the pH and thereby diminish the release of Cd from the colloids and the Cd uptake. Due to that it is liquid, KSi increases the pH fastest with 1 unit, while both Solaritt and Microsilica very slowly release their content and did not much change the pH of the soil in the field study. Since all three Si additives gave the same Cd effect, while the effect on pH differed it is most likely that Si has its own effect as well. The high pH was after two weeks down to the pH of the control soil in all parcels.

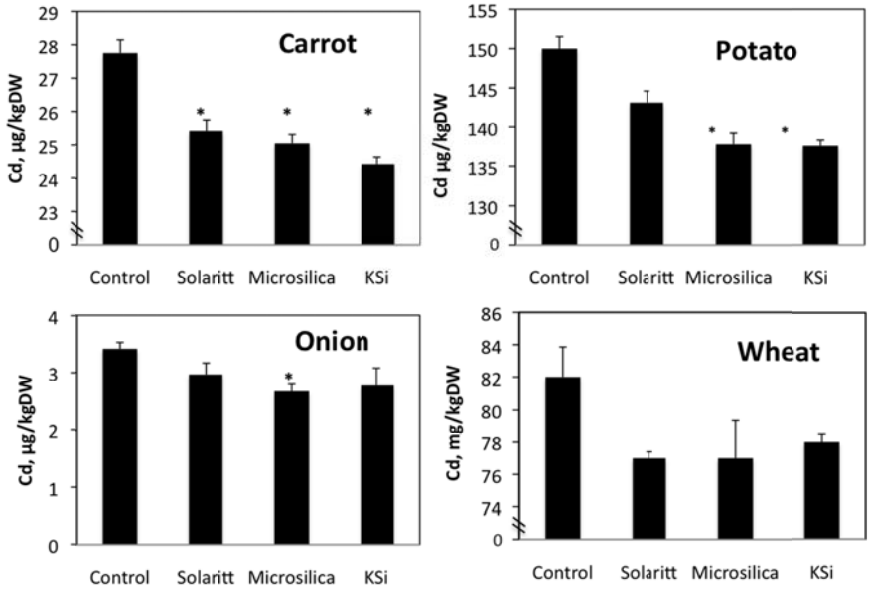

Fig. 1: Cadmium concentration in potato tuber, carrot, onion (µg/kg) and wheat grains (mg/kg) after cultivated in field in the presence or absence of silicon in form of liquid potassium silicate (KSi), amorphous (Microsilica) and powder form (Solaritt). n=4, ±SE. * significant different from control.

CONCLUSIONS

We can conclude that addition of silicon decreases Cd content in edible parts of plants. However, the addition of silicon as high as 500 kg per ha is quite high to get effect. The efficiency of silicon effect has therefore to be increased for economic reasons to be able to decrease the size of application of Si.

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Novel Method Developments for Speciation and Localization Analyses of Essential Trace Elements in Cereal Grains

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INTRODUCTION

The loading of essential trace elements like Zn, Fe, Cu and Mn into the cereal endosperm tissue is vital for improving the health status in populations, which depend on cereals in their diets. In this sense, not only the total grain and total endosperm concentrations are important, but also the localization, co-localization and speciation with other elements. Biofortification strategies offer different ways to optimize the concentration of essential trace elements in cereal grains, including agronomic and biotechnological ones. However, these strategies needs to be scientifically verified with respect to the exact localization, speciation and ultimately the bioavailability of the micro-nutrients. Here we present method developments for robust speciation analyses of the cereal endosperm. In addition, we will present our latest progress with multi-elemental bioimaging of cereal grains, using LA-ICP-MS.

METHODS

We use the combined strengths of chromatography and laser ablation in hyphenation with state-of-the-art elemental detection with ICP-QQQ-MS. For speciation studies we have developed a new pre-concetration method based on anion exchange chromatography, performed without online connection to the ICP-MS. Extracted and filtered samples of white rice were loaded onto a Strong Anion eXchange (SAX) chromatography column (HiTrap Q HP, GE Health care, Sweden) which retains negatively charged compounds. The column was hereafter eluted with an increasing ionic strength, and the different elution fractions were collected and analyzed for both for their total concentration and for their speciation, using a SEC column in hyphenation with ICP-MS. The ICP-MS was operated in MS/MS mode with oxygen as reaction gas, which enabled high sensitivity for the target elements Fe, Zn, Cu, Mn, S and P. Laser ablation-ICP-MS was used to analyze the localization and co-localization of the same elements in durum wheat grains. The surface of the grains were ablated for bio-imaging and localization analysis with ICP-MS, using the following laser settings: Energy 30%, Scan speed 80, Repetition rate 60 and Spot size 35 µM. The data points were related to ^{13}C and presented both as lane scans and contour plots.

RESULTS AND DISCUSSION

The speciation studies revealed that a majority of the water-extractable Zn and Fe was retained on a strong anion exchange chromatography column (SAX), hence existed as negatively charged compounds *in vivo*. When analysing the different elution fractions it was observed that the majority of Zn eluted at low salt concentration (0.2M SAX fraction), whereas the majority of Fe eluted at the high salt concentration (0.5M SAX fraction). The 0.2M SAX fraction also had the highest concentrations of S, Cu and Mn, which indicates that Zn, Cu and Mn are mainly bound to proteins, as has been shown also in previous studies (Persson et al. 2009). In the 0.5M SAX fraction, Fe eluted together with the majority of P, indicating binding to phytic acid. As a result of their different binding strength to the column, the majority of the extracted Fe and Zn could be physically separated already in this first chromatographical step. These observations were verified in the following SEC-ICP-MS analysis (Fig 1.). Apart from the convenient separation of Fe/phytic acid from Zn, which is very useful in successive analyses, the SEC chromatography was much more robust compared to SEC-ICP-MS analyses of crude extracts. The recovery from the column was close to 100% for all elements, which is to be compared with <50% recovery of Zn in the crude extracts.

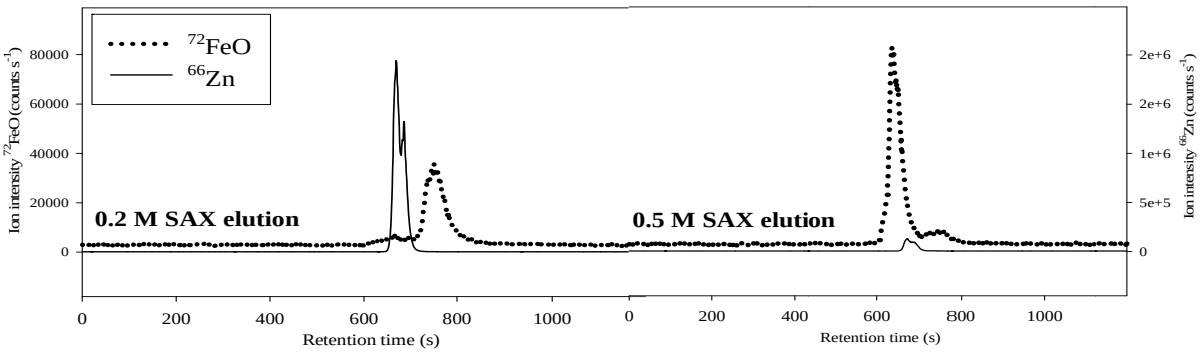

Fig. 1: SEC-ICP-MS chromatogram of rice endosperm extracts eluted from a SAX column with 0.2M salt and 0.5M salt, respectively, showing that the majority of Fe:P and Zn:S complexes can be physically separated.

Bio-imaging of the localization of essential trace elements were performed on durum wheat grains, grown in either low or high nitrogen, with either low, medium or high Zn fertilization, applied as foliar spray (Kutman *et al* 2010). The lane scans and the images clearly showed differences in endosperm concentrations, both with respect to the N and the Zn treatments (Fig.2). The multi-elemental pictures of S, P, Fe, Mn and Cu will be presented at the conference.

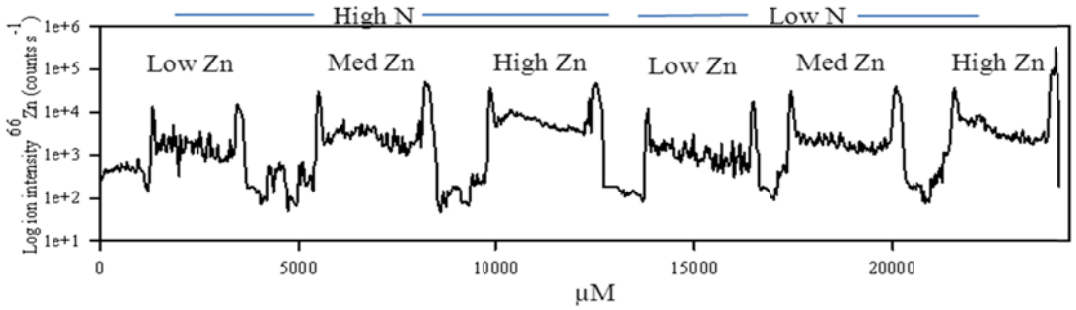

Fig 2: Lane scans of durum wheat grains with different N and Zn treatments, analysed with LA-ICP-MS.

CONCLUSIONS

The improvements in pre-concentration of cereal grain extracts pave the way for robust future speciation analyses of protein complexes with Zn, Mn and Cu. This will enable proper identification of both low and high abundant Zn/Mn/Cu binding proteins *in vivo*, which will be of great value for further improvement of the micro-nutrient density of cereal grain. Bio-imaging with LA-ICP-MS is a useful tool for proper evaluation of the elemental distribution *in situ*, which enables high-sensitivity and high resolution comparisons of different biofortification strategies aiming for improved loading of the cereal endosperm with essential trace elements.

ACKNOWLEDGEMENTS

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Biotic and Abiotic Reactions Influencing Iron Availability in the Rhizosphere

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INTRODUCTION

Iron (Fe) biofortification of plant-based food is a major challenge of world agriculture. Enhancing uptake form the soil can be regarded as a key step in this process.

Accumulation of Fe in plants strongly depends on the presence of available forms of the micronutrient in the soil and on the capacity of plant to take it up from non-readily soluble sources and counteracting the competition with soil microorganisms. Iron is relatively abundant in many cultivated soils and present in different species such as: 1) FeII in primary minerals, 2) FeIII in secondary minerals, as Fe crystalline minerals and poorly ordered crystalline (hydr)oxides, 3) soluble and exchangeable Fe and 4) Fe bound to organic matter in soluble or insoluble forms. Under aerated conditions and pH values above neutrality, the total concentration of inorganic Fe species in the soil solution is orders of magnitude lower than that required for an optimal growth of plants. Therefore, Fe deficiency is a frequent problem for many crops, particularly in calcareous soils.

Physical, chemical and biological processes occurring in the soil and, particularly in the rhizosphere, can affect Fe biogeochemistry and, in turn, the presence of the forms available for plants and microorganisms (Colombo et al., 2013).

RESULTS AND DISCUSSION

The solubilization of Fe from soil mineral sources is a slow process regulated by pH and redox potential (O_2 concentration) and by the dissolution–precipitation phenomena of both crystalline and poorly ordered Fe-hydroxide minerals. These processes are particualrly complex within the rhizosphere, where a competition for Fe occurs, due to the activity of living roots and microorganisms (exudation and nutrient uptake) (Roemheld and Marschner, 1986).

Iron-reducing bacteria can solubilize high amounts of FeII in waterlogged (paddy or poorly drained) soils. In aerate conditions, Fe-oxidizing bacteria can promote the oxidation of FeII- containing minerals. However, in cultivated soils, at neutral pH, FeII is subject to rapid chemical oxidation, and the produced FeIII quickly hydrolyzes and precipitates as Fe (hydr)oxides.

The principal means by which soil microbes acquire Fe relies on the synthesis and release of low molecular-weight Fe-binding molecules called siderophores (MSs) (Lemanceauet et al. 2009). The FeIII–MSs complexes are characterized by stability constants much higher than those measured for Fe complexes formed by organic compounds frequently present in the rhizosphere like anions of organic acids (e.g. oxalate or citrate) or phytosiderophores (PSs) released by grass roots. MSs can mobilize Fe from minerals by a complexation reaction, acting alone or in combination with simple carboxylic acids (e.g. oxalate) and organic reducing agents. MSs may also remove Fe from organic complexes (like Fe complexed by organic acids, phenols and soil humic substances, HS) via ligand exchange.

Plants have evolved uptake mechanisms to acquire Fe that differ between dicots (including also non-graminaceous monocots) and monocots (Kobayashi and Nishizawa 2012). In the first case, Fe is acquired by a reduction-based mechanisms that is associated to the release of protons (rhizosphere acidification), organic acids and phenols. In graminaceous species Fe uptake is based on chelation of FeIII to PSs, strong ligands belonging to the mugineic acids' (MA) family. These ligands are released into the rhizosphere where they form FeIII-PS complexes that are then taken up by a specific transporter. Rice plants possess a mixed strategy as they release PSs but can also take up Fe^{2+} with an obvious advantage in wetland cultivation system. Increasing synthesis and release of PSs has been proven to be a promising strategy to improve Fe acquistion in graminaceous plants.

FeIII–PS complexes might also serve as a substrate for reduction or even be absorbed directly by roots of neighbouring intercropped dicots. PSs can promote dissolution of Fe (hydr)oxides in synergy with organic acids, and mobilize FeIII from different sources, including HS, by a ligand-exchange mechanism. This is unlikely to occur with FeIII-MSs, due to their very high stability constant; however this process has been reported for some MSs. Furthermore direct uptake of specific FeIII-MSs complexes has been suggested for dicots. The way by which plants and microbes take up Fe (as intact FeIII-complex or ionic Fe^{2+}) can affect weathering of Fe minerals, due to the variable persistence of chelating compounds in the rhizosphere.

Root and microbial respiration can contribute to rhizosphere acidification; this can be achieved also by the release of carboxylates coupled to the activation of the PM H^+-ATPase or root cells. Acidification would lead to a higher availability of soluble Fe in the rhizosphere and favor the operation of Fe uptake mechanisms (e.g. FeIII reduction). Compounds released by plant roots, such as carboxylates and, as recently evidenced, phenols can contribute to mobilize Fe from unavailable sources forming complexes that are the major part of soluble Fe in the soil; however their efficiency is strongly dependent on the soil conditions (pH and redox status). Molecular mechanisms of root exudation and the reactions of exudates with Fe-bearing minerals are to be elucidated. These achievements are now complemented by accurate and more realistic analyses of the amount and type of the released molecules.

Mutual relationships between roots and microbes have been described. In fact root exudates can selectively shape the rhizospheric microbial community with impact on the production of MSs and auxins. Rhizospheric microbes can, in turn directly and/or indirectly affect Fe acquisition by roots. Bacteria can use organic exudation as carbon and energy source promoting organic matter turnover in the rhizosphere; more refractory molecules are used to generate complex HS. These latter are not readily usable by soil microorganisms, but, due to their complexing and redox properties, they strongly affect Fe dynamics and the amount of Fe available for plant and microbial uptake; furthermore direct effects on plant uptake mechanisms have been reported.

CONCLUSIONS

Understanding the mutual interactions among plant, microbes and soil minerals is crucial for the development of new agronomic tools and strategies for the genetic improvement of crops, aiming at maximizing Fe use efficiency from the soil. New analytical and molecular techniques are promising approaches to achieve the goal. Furthermore, intercropping and the use of natural Fe-complexes might represent suitable tools for a sustainable management of Fe plant nutrition.

ACKNOWLEDGEMENTS

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Role of Nitrogen Nutrition in Root Uptake, Leaf Penetration, and Grain Accumulation of Iron in Wheat Plants

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INTRODUCTION

Iron (Fe) deficiency is a well-documented problem in cultivated soils and responsible for impairments in yield capacity and nutritional quality of crop plants, especially in alkaline soils (Borg et al., 2009). Cereal grains are inherently low in concentrations of total and bioavailable Fe to meet human requirements. Application of soil and/or foliar Fe fertilizers might be a solution to improving Fe concentrations of cereal grains. However, when applied to soils, Fe is often rapidly converted to poorly soluble forms, and its acquisition by roots is limited (Marschner and Römheld, 1994). Foliar applications of Fe fertilizers also remain less effective in terms of increasing grain Fe concentrations, probably due the poor penetration through the leaf cell walls and limited phloem transportation of Fe. Published data show that enhancements in the nitrogen (N) nutritional status of plants result in significant increases in root uptake, transport and grain allocation of Fe (Aciksoz et al., 2011b). The reason of this N effect is not fully clear. At least one of following mechanisms contribute to N-induced grain accumulation of Fe: i) improving root absorption capacity, ii) facilitating transport within plants and into seeds and iii) increasing seed/grain sink capacity (by enhancing metal-binding proteins) for Fe. Alternatively, adding urea to inorganic or chelated forms of foliar Fe fertilizers at 1% (*w/v*) had a positive impact on increasing grain Fe concentrations in wheat (Aciksoz et al., 2011a). Urea can stimulate leaf penetration and translocation of iron within wheat plants. Urea is reported to easily permeate through the cuticular membranes (10 to 20 times better than inorganic ions) (Wojcik, 2004). This study has been conducted to study the impact of soil and foliar leaf-applied nitrogen on shoot and grain accumulation of Fe. In case of foliar treatments, radiolabelled Fe (^{59}Fe) has been applied to leaf tips with and without urea, and translocation (partitioning) of the leaf-treated ^{59}Fe has been studied in the whole plant.

METHODS

Soil and hydroponic culture experiments were conducted using durum wheat (*Triticum durum* cv. Balcali2000) to clarify the interrelationship between N nutrition and Fe accumulation in grain. Experiments focused on the effect of varied N concentrations in i) growth medium (soil and hydroponic culture) and ii) vegetative tissue (foliar application) on root uptake, transport and seed deposition of Fe. Attention was given to root uptake, root-to-shoot transport, retranslocation from vegetative tissues and seed deposition of radiolabelled Fe (^{59}Fe)/Fe in plants treated differently by N fertilization. Analysis of ^{59}Fe activity in the plant samples was realized by using a Perkin Elmer 2480 WIZARD2 Automatic Gamma Counter, and measurement of mineral nutrients were realized by using inductively coupled plasma optical emission spectrometry (ICP-OES). Nitrogen concentration in the samples was determined by using a LECO Tru-Spe C/N Analyzer.

RESULTS AND CONCLUSIONS

The studies demonstrated that increasing soil N fertilization enhanced both shoot and grain Fe concentrations, while soil-applied Fe fertilizers remained without effective on grain Zn. The form of N fertilizers had no effect on shoot Fe. Foliar Fe fertilization in different forms such as FeEDTA, FeEDDHA, $FeSO_4$ had very slight positive effect on grain Fe. However, adding urea in the foliar Fe fertilizers enhanced grain Fe concentration regardless of Fe forms. Similarly, in the studies where 59FeEDTA was applied onto 5 cm long tips of flag leaves (by soaking intact flag leaves into ^{59}FeEDTA solution), inclusion of urea in the ^{59}FeEDTA solution substantially increased penetration of ^{59}Fe into the foliage and translocation of ^{59}Fe from flag leaves to the grain (Table 1). The proportion of ^{59}Fe found in grains increased from 8.8 to 34.6% by including urea in the treatment solution, but such an enhancing urea

effect was not observed for ^{59}Fe translocation into shoots (Table 1). This result implies that transport of leaf-applied Fe into grains is, at least partly, a sink-driven process because developing grains are better competitors than shoots at the reproductive growth stage (Marschner, 2012).

Table 1. Effect of increasing urea concentration in the ^{59}FeEDTA treatment solution on the activity of ^{59}Fe in the treated flag leaves, shoot (straw) and grain, and on the relative distribution of absorbed ^{59}Fe to the grain and straw of the mature durum wheat plants.

Urea concentration in ^{59}FeEDTA solution	^{59}Fe activity			Relative proportion of absorbed ^{59}Fe	
	Treated flag leaf	Remainder of shoot	Grain	Grain	Shoot
(% w/v)	(CPM)			(%)	
0	3224	71	265	8.8	2.4
0.2	2929	62	584	22.2	2.1
0.4	4494	93	1485	34.6	2.2
0.8	7507	116	1907	28.1	1.6
CV (%)	27	29.8	37.3	41.6	39.9
F test	**	**	**	**	n.s.
$LSD_{0.05}$	608	27	456	11.6	-

The results clearly indicate that N nutritional status of plants has positive impact on root uptake, shoot accumulation and grain deposition of Fe. Foliarly applied- urea also facilitated penetration of Fe into leaf and translocation of Fe from the treated leaf to the other plant parts such as grains. Most probably, N nutritional status of plants greatly affect activity and pool of compounds involved in Fe transport and chelation such as Fe transporter protein and nicotianamine. The results suggest that urea inclusion into foliar Fe treatment solutions represent a useful agronomic practice for biofortification of cereal grains with Fe.

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The Effect of Sulphate Fertilization on Zinc Uptake and Accumulation in Wheat Grains

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INTRODUCTION

Zinc deficiency in human populations caused most often by inadequate dietary intake is recognized as a global nutritional problem. Cereal crops such as wheat represent a major dietary source of micronutrients, especially in low-income countries. Increasing Zn concentrations of wheat grains is therefore an important global challenge.

METHODS

This study investigated the role of sulphur, nitrogen and zinc nutrition on wheat (*Triticum aestivum*) Zn status and grain concentration. Plants were grown in pots in a climate chamber at varied levels of sulphur (0, 25, 50 and 100 mg SO_4-S kg^{-1} dry soil), nitrogen (100 and 200 mg NO_3-N kg^{-1}) and zinc (0 and 5 mg Zn kg^{-1}), harvested at maturity and analysed for element concentrations in the grains and the shoots. The soil used in the experiment was classified as a sandy clay loam according to the US soil taxonomy and originated from Switzerland. The biomass harvest index (BHI), the percentage of grain biomass compared to the biomass of the whole plant and the zinc harvest index (ZHI) were calculated as indicators of the plants' efficiency to build proteins or to accumulate zinc in the grains, respectively.

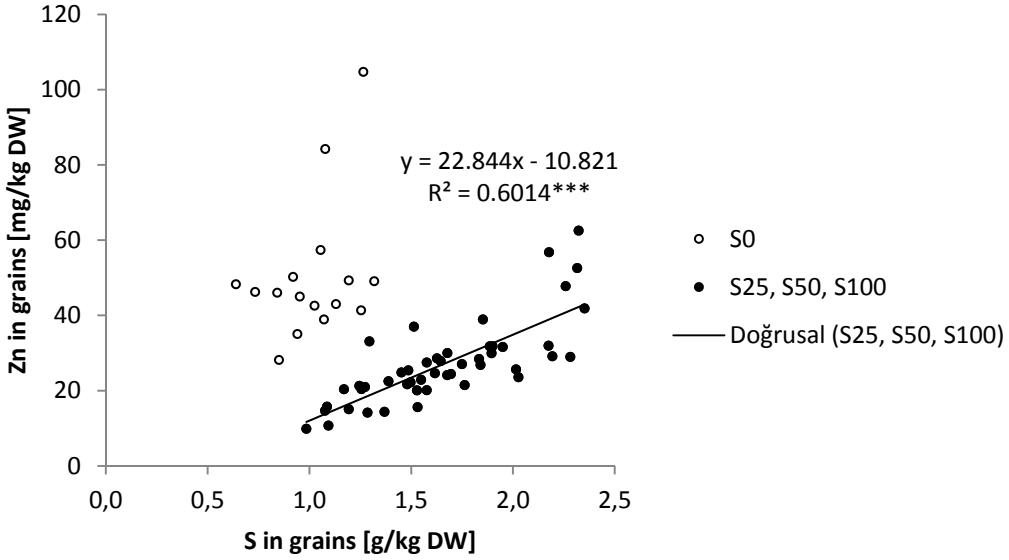

Fig. 1: Zinc concentrations plotted against sulphur concentrations in the grains. For the regression ($p < 0.001$), the plants under the S0 treatment were excluded from the data because of dominant sulphur deficiency effects.

RESULTS AND DISCUSSION

Where no sulphate was added (S0), wheat plants were strongly S deficient while N was the limiting nutrient in the other treatments (S25, S50 and S100). Zn was sufficient in all the treatments.

When sulphur supply was adequate, the Zn and S concentrations correlated positively in the grains (R^2=0.601) (see **Figure 1**). The relatively constant S:Zn ratio in the grains confirmed this proportional

behaviour of S and Zn accumulation in wheat grains. As shown before by Erenoglu et al. (2011) and Kutman et al. (2011), Zn and N concentrations in the grains were also positively correlated (R^2=0.599).

Maximum ZHI (see **Figure 2**) and BHI values occurred at N:S ratios (by weight) in grains around 13:1 to 15:1. This is close to the ratio of 15:1 required for protein synthesis as stated by Zhao et al. (1999).

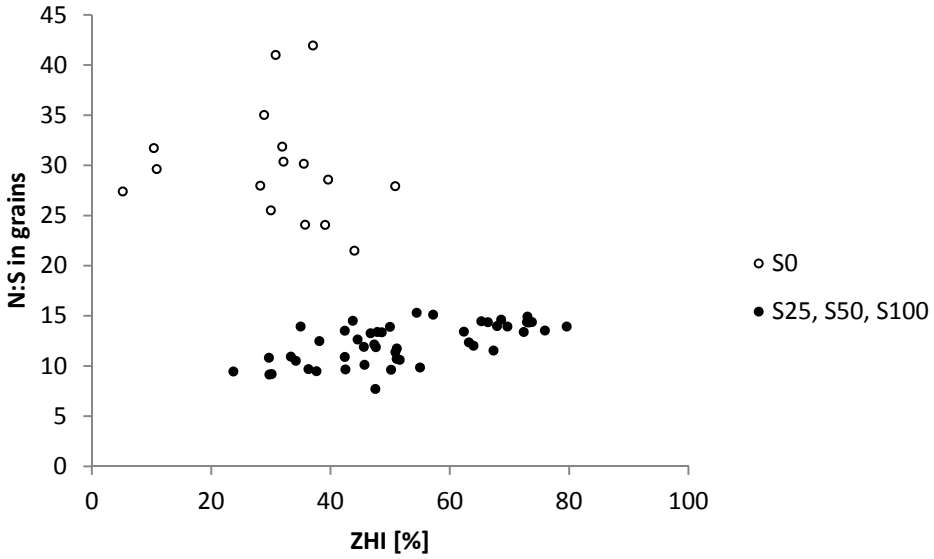

Fig. 2: N:S ratio in the grains (by weight) plotted against the zinc harvest index (ZHI).

CONCLUSIONS

The investigation showed that Zn accumulation in wheat grains is not only affected by the nitrogen metabolism but also by the sulphur metabolism of wheat plants and most importantly by the balance between those two nutrients. N:S ratios (by weight) around 13:1 to 15:1 indicated a good balance between N and S nutrition for protein synthesis. Zinc accumulation in wheat grains was closely linked to the efficiency of a wheat plant to synthesize proteins. According to these findings, the grain N:S ratio is a better indicator for wheat Zn accumulation than the concentrations of nitrogen or sulphur alone.

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Zinc Biofortification of Wheat through Preceding Crop

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INTRODUCTION

Zinc deficiency is widespread among human populations worldwide. Agronomic biofortification strives to increase micronutrient density in edible parts of food plants by agricultural methods of crop cultivation, in particular by fertilizer application (Cakmak, 2008; Graham et al., 2001). The hypothesis of this study was that also the cultivation of preceding crops (or shortly: precrops) and the incorporation of their residues into soil has potential to increase grain Zn density without compromising yields. To test this hypothesis we performed a field experiment in which we assessed the effects of different precrop treatments on the availability of soil Zn and on the yield, Zn concentration and phytic-acid-to-Zn (PA:Zn) molar ratio of the grains in subsequent wheat cultures.

MATERIALS AND METHODS

The study was conducted over two subsequent growing seasons on two fields of the IUT Agricultural Research Station at Rudasht (32, 29/N, 52, 10/E) in central Iran. The soils of the two experimental fields were severely deficient in DTPA-extractable Zn (average concentrations: 0.15 mg kg^{-1} and 0.18 mg kg^{-1}).

The following four plant species/cultivars were used as precrops: sunflower (*Helianthus annuus* L. cv. Allstar), sudan grass (*Sorghum bicolor* L. cv. Speed Feed), clover (*Trifolium pretense* L.) and safflower (*Carthamus tinctorius* L. cv. Koseh-e-Isfahan). Plots with no precrop (fallow) were established as control treatment.

After land preparation, fertilizers were applied based on soil testing in accordance with the recommendations given by the Iranian Soil and Water Institute (ISWI) (Milani et al., 1998). After harvesting the aboveground parts of the precrops were air-dried, crushed to pieces of 0.5-2 cm size and incorporated back into the soil of the respective plots on which they had been grown. The application rate was 7 t/ha, which is the average dry matter yield for the precrops used in this experiment in the study area. Three weeks after residue application, wheat (*Triticum aestivum* L. cv. Back Cross) was planted on 12 December 2009 in the first experimental year and on 20 November 2010 in the second year. Harvest was on 23 June 2010 in the first year and on 9 July 2011 in the second year. Wheat yields were determined from the plants harvested in the central 1.0 m^2 of each plot.

Samples of the harvested precrop and wheat plants were analyzed for Zn. In addition, wheat grains were analyzed for phytic acids (PA) using a modified version of the Makower method (Makower, 1970).

RESULTS AND DISCUSSION

Using clover as preceding crop resulted in the largest increase in the grain yield of the subsequent wheat culture. In contrast, grain yields were lower in the sorghum and safflower treatments than in the fallow treatment. This might have been due to some degree to allelopatic effects. All precrop treatments significantly increased the grain Zn concentration of wheat. The largest increase was found with 93.6% in the clover treatment. Higher grain Zn concentration of wheat plants grown after clover could partly be due to the positive effects of clover residue on soil availability of Zn. It seems that organic acids produced from decomposition of clover residues have higher ability of forming soluble complexes with Zn in soil than those produced from the residues of other preceding crops. Schulin et al. (2008) reported that plant residue decomposition can produce ligands that form soluble complexes with Zn and thereby bring Zn bound to solid phases into solution. All precrops significantly decreased the PA:Zn molar ratio of the grains. The largest decrease was found with 48% in the clover treatment. Only in this treatment it decreased to values lower than 15. This result shows that using clover as a precrop for green manure

cannot only increase grain Zn density, but at the same time also enhance the availability of this Zn source in human nutrition and all this with increased yield.

CONCLUSION

Incorporation of precrop residues into soil can significantly increase grain Zn concentrations of subsequent wheat cultures and at the same time also increase its nutritional availability for human consumers. The magnitude of these effects depends on the type of precrop. Clover led to the largest increase in grain Zn density as well as decrease in PA:Zn molar ratio. It was also the only precrop that increased yield.

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Biofortification in *Triticum aestivum* – Nutrients Accumulation and Stability

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INTRODUCTION

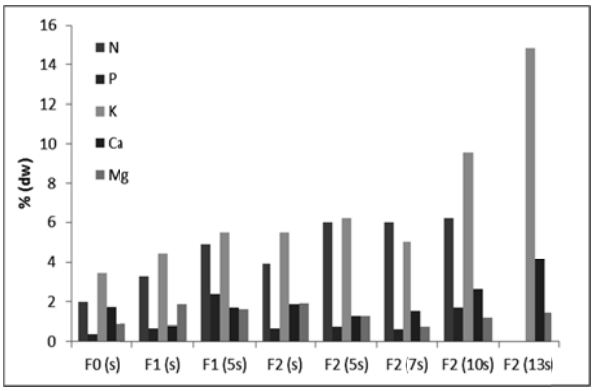
Although deficiencies of various micronutrients are common issues in the human societies that can lead to public health difficulties, biofortification of staple foods can overcome this problem. In this context, this work aims to assess, in environmental controlled conditions, the limits of nutrients uptake and translocation plasticity and elasticity in Zn and Fe biofortified *Triticum aestivum*.

METHODS

Parental (F0), F1, F2, F3 and F4 generation seeds of *Triticum aestivum* L. cv Roxo were sown in a walk-in growth chamber, under environmental controlled conditions (80% RH; 24/20^{o}C day/night temperatures; PPFD of *ca*. 700 µmol m^{-2} s^{-1}, 12 h photoperiod). F0 (s), F1 (s), F2 (s), F3 (s/s), F3 (7s/s, 10s/s, 13s/s) and F4 (s/s) plants were irrigated with a standard solution until harvest. F1 (5s, 7s, 10, 13s) and F2 (5s, 7s, 10, 13s) pot seeds were irrigated with a standard solution (s) during 1 month after germination, and thereafter with 5, 7, 10 and 13 fold nutrients concentrations until harvest. The seeds: F3 (s/s, s/5s) and F4 (s/s) were obtained from F2 (s) and F3 (s/s) plants grown with standard solutions, respectively; F3 (5s/s, 5s/5s), F3 (7s/s), F3 (10s/s) and F3 (13s/s) were obtained from F2 plants grown with 5, 7, 10 and 13 fold higher concentrations, respectively; F4 (5s/5s) were obtained from F3 (5s/5s) plants grown with 5 fold higher concentrations of all nutrients. F3 (s/5s), F3 (5s/5s) and F4 (5s/5s) pot seeds were irrigated with a standard solution (s) during 1 month after germination, and thereafter with 5 fold nutrients concentrations until harvest. In the grains: N content was measured by the Kjeldahl method (Nitrogen Analyser Tecator 1002, Nitrogen Digester Tecator 2006); P by the ascorbic acid method, after acidic digestion of the sample (Spectrometer UV/Vis Cecil 9000); the other elements by incineration at *ca*. 550^{o}C followed by nitric acid digestion (Unican model 939 absorption unit).

RESULTS AND DISCUSSION

Between parental F0 (s) and F1 (s) and F2 (s) generation seeds of bread wheat cv Roxo, the levels of (Fig. 1, 2): Zn increased 7 and 10 fold; Fe increased about 1.35 and 4 fold; N, P, K, Mg, Mn and Na increased progressively. Additionaly, between F0 (s), F2 (s) and thereafter F2 (13s) the amounts of (Fig. 1, 2): Fe increased about 4 and 10 fold; Zn augmented 5 and 10 fold. Besides, between F0 (s) and F2 (s, 5s, 7s, 10s and 13s), the contents of N, P, K, Cu, Mn and Na increased progressively (Fig. 1, 2). Accordingly, from F0 to F2 generation seeds of *Triticum aestivum*, the applied technique for biofortification was coupled to a general increase of all nutrients.



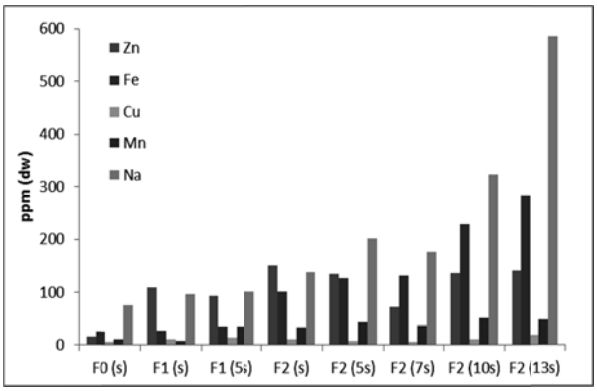
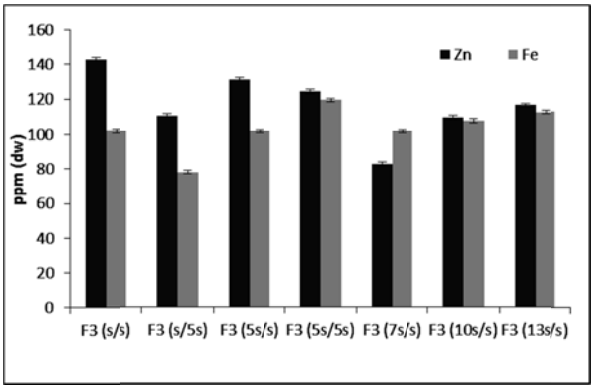


Fig.1 and 2. Nutrients accumulation in wheat seeds belonging to F0, F1 (s, 5s) and F2 (s-13s).

Between F2 (s and 5s) and F3 (s/s and 5s/5s**)** the levels of Zn and Fe did not vary significantly (Fig. 3), suggesting that a maximum of these nutrients accumulation was reached without inducing toxicity. Moreover, between F3 (s/s) and F3 (s/5s) a significant decrease was found for Zn and Fe accumulation in the seeds (Fig. 3), which indicates that the chemiosmotic mechanism of these mineral ions in the plasma membranes had limited capabilities to metabolise higher amounts of nutrients (i.e., triggers nutritional misfit, possibly because the induction of root-based transport systems became inhibited, eventually at the level of the root architecture). Moreover, the pattern found (Fig. 3) between F2 (7s, 10s and 13s) and F3 (7s/s, 10s/s and 13s/s), strongly points that cation exchange was significantly affected mostly due to lower levels of nutrients in the growth medium. Still, between F2 (5s) and F3 (5s/s) the values for seeds accumulation remained consistently similar (Fig. 2, 3), which suggests that the unidirectional influx of Zn and Fe ions and translocation to the shoots is a carrier-mediated uptake system. Between F3 (s/s and 5s/5s) and F4 (s/s and 5s/5s) the amounts of Zn and Fe remained similar (Fig. 4), thereafter showing a consistent stability of nutrient-root influxes.



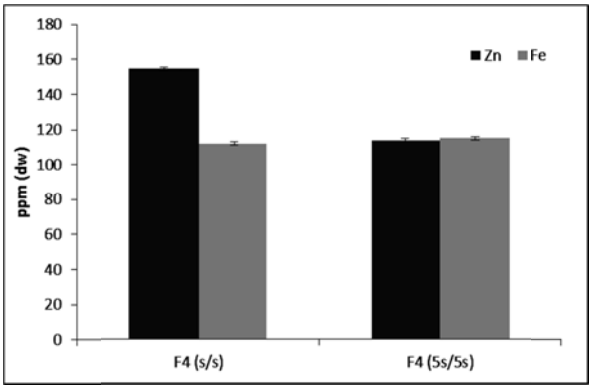


Fig.3. Zn and Fe contents in F3 wheat seeds. **Fig. 4.** Zn and Fe contents in F4 wheat seeds.

CONCLUSIONS

Supplementing all macro and micronutrients proportionaly, between 2 sucessive generations, significantly increases the amount of nutrients in *Triticum aestivum* L. seeds. From the 3rd progeny onwards, if adequade or 5 times higher amounts of all nutrients are provided in the nutrient solution, a plateau is achieved for Fe and Zn contents in the seeds, it can be concluded that both nutrient solutions can be used in biofortification studies, without reaching the threshold of toxicity. Moreover, if plants adapted for two generations to 5 times higher levels of all nutrients are thereafter submited to lower concentrated nutrient solution, the rate of Zn and Fe consistently persist, which suggest that a metabolic plasticity for ions uptake and/or translocation to the shoot is developed.

Biofortification in *Triticum aestivum* – Distribution of Nutrients in the Grain

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INTRODUCTION

Metals availability for plant uptake is driven by complex interactions between the chemical properties of cations, the composition and physicochemical properties of the soil, microbial activity and plant roots (Antunes et al., 2006). Additionally, although Zn and Fe biofortification of plants is dependent on the size of plant-available nutrient pools in soils, its accumulation and distribution in the grain is dominated by the phloem sap system (Graham, and Stangoulis, 2003). In biofortified bread wheat (*Triticum aestivum* L.), grown in environmental controlled conditions, this work aimed to assess the distribution of micro and macro nutrients in the grain.

METHODS

Parental (F0), F1 and F2 generation seeds of *Triticum aestivum* L. cv Roxo were sown in a walk-in growth chamber, under environmental controlled conditions (80% RH; 24/20°C day/night temperatures; PPFD of *ca*. 700 μmol m^{-2} s^{-1}, 12 h photoperiod). F0 (s), F1 (s) and F2 (s) were irrigated with a standard solution until harvest. F1 (5s, 7s, 10, 13s) and F2 (5s, 7s, 10s, 13s) seedlings were irrigated with a standard solution (s) during 1 month after germination, and thereafter with 5, 7, 10 and 13 fold nutrients concentrations until harvest. Scanning electron microscopy studies, considering duplicates, were performed on transversal and longitudinal sections (Fig. 1) of F0 (s) and F2 (s, 5s, 7s, 10s and 13s) wheat grains, using a Jeol 330 coupled with a X-ray microanalyser Tracor Northern Series II.

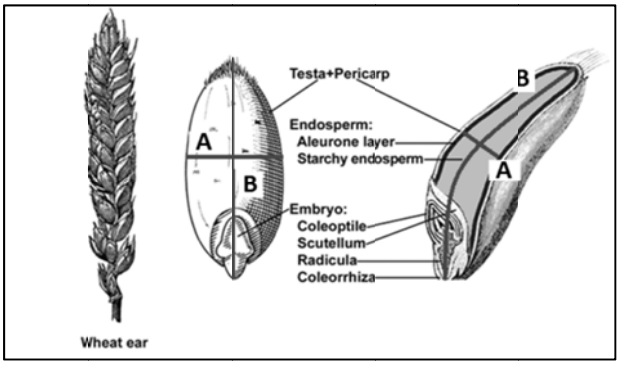

Fig. 1. Transversal (A) and longitudinal (B) sections selected for scanning electron microscopy in the cereal grains and X-ray microanalysis.

All analysis were performed at 25 Kv, 35x magnification, using a static beam spot under standardized conditions. Peak to background ratios (K ratios) were calculated using the routine supplied by the manufacturer. The LineScan analysis was done along a transect in the grain, with the following constant conditions - Number points (1000), dwell time (msecs), image size (512x512).

RESULTS AND DISCUSSION

Scanning electron microscopy coupled with X-ray microanalysis indicated that, in the starchy endosperm of the equatorial region, Fe and Zn showed minimum values in F2 (s), F2 (5s) and F2 (7s), but the ratio

Fe/Zn increased between F2 (s) and F2 (5s) (with minimum values in the centre) (Table 1). Moreover, the longitudinal analysis showed that (Table 1) in: F0(s) Fe deposits mainly in the embryo region, whereas the oposite occurs with Zn; F2 (s), Fe and Zn predominates in the centre; F2 (5s) and F2 (7s) Zn prevails in the embryo, the opposite occurring with Fe.

Distribution in the starchy endosperm of the equatorial region was found to be heterogeneous in F0 (s), F2 (7s) and F2 (10s) for (P, Ca, Fe and Zn), (K, Mg and Cu) and (P) respectively; to prevail in the centre for F0 (s), F2 (s) and F2 (13s) for (k), (K and Mg) and (K), respectively; to overcome in the periphery of F2 (5s), F2 (7s), F2 (10s) and F2 (13s) for (k), (P), (K) and (P), respectively.

Considering the longitudinal section of the grains, the distribution of Mg, P, K, Ca, Mn and Cu, between the embryo and the periphery, was found to be homogeneous in F0 (s), whereas in F2 (s), F2 (5s) and F2 (7s) prevailed in the centre.

Table 1. Spectral analysis of Fe and Zn deposition in the grain (%).

		F0 (s)	F2 (s)	F2 (5s)	F2 (7s)	F2 (10s)	F2 (13s)
		Equatorial Region					
Fe	*Periphery 1*	4.63	6.01	3.60	0.91	5.11	5.51
	Centre	5.72	4.09	5.05	1.53	6.35	8.96
	Periphery 2	5.34	5.21	5.80	2.04	6.92	5.83
Zn	*Periphery 1*	6.90	5.30	3.95	1.05	7.26	8.44
	Centre	7.59	6.23	6.58	1.08	8.29	11.8
	Periphery 2	7.93	6.04	4.58	0.95	3.80	9.90
Fe/Zn	*Periphery 1*	0.67	1.13	0.91	0.87	0.70	0.65
	Centre	0.75	0.66	0.77	1.42	0.77	0.76
	Periphery 2	0.67	0.86	1.27	2.15	1.82	0.59
		Longitudinal Region					
Fe	*Periphery 1 (embryo)*	2.20	0.39	0.60	8.38		
	Centre	1.38	3.03	0.64	6.38		
	Periphery 2	0.90	0.575	1.02	6.58		
Zn	*Periphery 1 (embryo)*	1.86	0.615	9.8	12.6		
	Centre	1.59	6.48	4.89	9.45		
	Periphery 2	2.87	1.52	1.24	9.76		
Fe/Zn	*Periphery 1 (embryo)*	1.19	0.634	0.061	0.66		
	Centre	0.87	0.467	0.13	0.675		
	Periphery 2	0.31	0.378	0.82	0.67		

CONCLUSIONS

In the biofortified grains (*i.e.,* F2), Zn and Fe prevail in the embryo and opposite periphery, respectively, with the starchy endosperm of the equatorial region having low accumulation of both nutrients. Moreover, Mg, P, K, Ca, Mn and Cu accumulation prevail in the starchy endosperm.

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Session-II:

Molecular Biology, Genetics and Breeding

Dissecting the Management of Essential and Toxic Metals in Plant Cells using NRAMP Transporters

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INTRODUCTION

Essential metal nutrition, metal toxicity and toxic metal accumulation are entangled processes. Most often, toxic metals enter and are distributed inside plants through the pathways that evolved to capture essential metals from the environment (Clemens et al., 2013). Metal toxicity often results from competition between essential and toxic metals. However, to improve essential metal availability in plant derived food for biofortification or to increase plant ability to accumulate and tolerate toxic metal for phytoremediation, it is necessary to control independently the accumulation of essential and toxic metals.

Most NRAMP tranporters have the ability to transport essential metals, such as iron (Fe) and manganese (Mn), as well as toxic metals, such as cadmium (Cd). In plants, this property was initially reported for AtNRAMP3 and AtNRAMP4 transporters (Thomine et al., 2000). Recently, several studies have demonstrated that OsNRAMP5, which is essential for Mn acquisition, is the main route for Cd uptake in rice (Clemens et al., 2013). AtNRAMP3 and AtNRAMP4 have redundant physiological roles in Fe and Mn remobilisation from vacuolar stores (Lanquar et al., 2005; Lanquar et al., 2010). Loss of their function also leads to strong Cd hypersentivity (Oomen et al., 2009; Molins et al., 2013). We have used genetics to identify the determinants of metal selectivity in NRAMP proteins and delineate functional networks involved in Fe storage in seeds, on one hand, and Cd toxicity and accumulation, on the other hand.

STRATEGIES AND RESULTS

Separating iron from cadmium transport function in NRAMP transporters

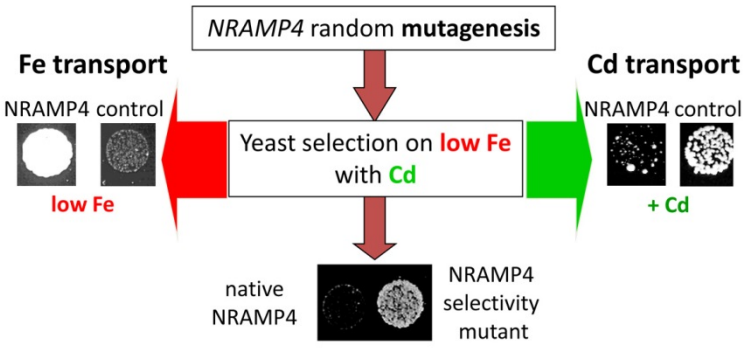

Fig. 1: Strategy to screen for mutations that alter NRAMP ability to transport Cd while maintaining Fe transport. AtNRAMP4 expression rescues growth of the Fe uptake mutant yeast strain *fet3fet4* (red arrow) but confers increased Cd sensitivity in yeast (green arrow). The screen selects NRAMP point mutants that rescue *fet3fet4* growth in presence of Cd.

To identify residues involved in Cd transport by NRAMPs, we performed a screen for mutations in the AtNRAMP4 coding sequence that maintain its ability to rescue growth of the yeast mutant *fet3fet4* impaired in Fe uptake but suppress the hypersensitivity to Cd induced by native AtNRAMP4 expression (Fig. 1). AtNRAMP4 cDNA was randomly mutagenized by error-prone PCR. Screening of about 50 000 mutants recovered 12 independent mutant *AtNRAMP4* cDNAs that allow growth on low iron medium supplemented with 10 µM Cd in the *fet3fet4* background. The screen recurrently selected mutations at four positions. Interestingly, several mutations affect residues that are conserved in plant and animal

NRAMPs. We demonstrated that the involvement in selectivity of one of them is conserved in an animal NRAMP transporter. Moreover, we showed that expression of AtNRAMP4 mutants selectively modulates Cd^{2+} accumulation in *Arabidopsis thaliana* roots.

Looking for genes involved in seed iron storage and cadmium tolerance in Arabidopsis thaliana

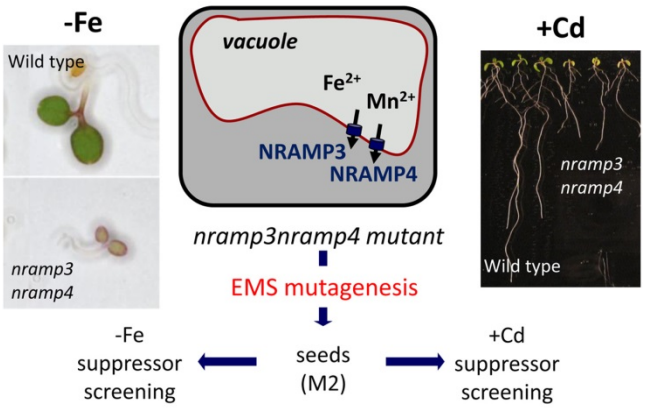

Fig. 1: Twin screens for mutations that restore germination on low Fe or that decrease Cd sensitivity in *nramp3nramp4* mutants.

To identify genes involved in Cd tolerance and Fe storage in seeds, we have set up twin genetic screens taking advantage of the strong phenotypes of *nramp3nramp4* double knockout mutants. On the one hand, we screened for mutations that rescue *nramp3nramp4* early development on low Fe (Fig. 2, left panel). On the other hand we screened for mutations that alleviate *nramp3nramp4* hypersensitivity to Cd (Fig. 2, right panel). The first screen isolated 50 candidate mutants. Among them, we already identified mutations in *AtVIT1*, which is known to mediate Fe storage in embryo vacuoles (Kim et al., 2006). Two mutants from the Cd tolerance screen were studied in detail. Both mutations partially restore Cd tolerance, but only one also rescues growth on low Fe medium. The genes affected by the mutations were identified by New Generation Sequencing. They highlight the importance of intracellular trafficking and root structure for Cd tolerance and accumulation.

Our results identified mechanisms involved in Cd transport and tolerance at the molecular, cellular and whole plant levels.

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Identification of the Genes Differentially Regulated in AtHMA4-Transgenic Tobacco Plants Exposed to 0.25 µM Cd

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INTRODUCTION

HMA4 is a transmembrane protein exporting Zn and Cd from the cytoplasm to the apoplast. It is expressed in cells surrounding xylem vessels and involved in the control of root-to-shoot translocation of Zn and Cd**.** Transgenic tobacco (*Nicotiana tabaccum*) plants expresing *AtHMA4* under CaMV 35S promoter displayed changed Zn and Cd root/shoot concentration and distribution (Siemianowski et al., 2011, Siemianowski et al., 2013). The extent of differences of the studied parameters depended on Zn and Cd concentration in the medium. Interestingly, exposure of 4,5-week-old tobacco plants to 0.25 µM Cd for 4 days resulted in reduced Cd concentration in roots and shoots.

The presented work contributes to understanding the consequences of the expression of *AtHMA4* at the molecular level. The aim of our study was to identify endogenous tobacco metal-homeostasis genes modified due to expression of *AtHMA4,* by comparison of the wild-type (WT) and transgenic plants exposed to 0.25 µM Cd for 11 days with SSH method*.* Considering the existence of the Zn-Cd-Fe cross-homeostasis network, identified genes will be used as markers for studying further their involvement in the generation of a plant response to varying levels of Zn and Cd in the medium.

METHODS

General Growth Conditions

To grow plants the ¼ Knop's solution was used as the basic control medium. Seedlings (3-week old) of WT and two homozygous *AhHMA4*-expressing lines (nos FL5 and FL9) grown on agar-solidified control medium were transferred to liquid medium for 1 week. Then the plants were further grown at the basic nutrient sollution suplemented with 0.25 µM Cd for 11 days. At the end of the experiments, plants were harvested and their respective parts were colected for RNA isolation for Suppression Subtractive Hybridization (SSH).

Suppression Subtractive Hybridization (SSH)

To identify genes differerntially regulated in *AtHMA4*-expressing plants subjected to long-term Cd treatment, the SSH method was applied. The subtracted libraries containing genes up-regulated and down-regulated in transgenic plants (in comparison to WT plants) were generated from the cDNA synthesized on RNA isolated from the apical (2,5 cm long) parts of roots of WT and transgenic plants (line 5) exposed to 0.25 µM Cd. The PCR-Select Subtractive Hybridization Kit (Clontech) was used according to the manufacturer's protocol. Subtracted cDNA fragments were cloned to pGEM vector. Duplicated membranes with spoted, PCR amplified subtracted cDNA fragments were hybridized with equivalent amounts of the probes labelled with digoxigenin (DIG) generated from forward- and reverse subtracted cDNAs. Clones that were confirmed in this screen to be differentially expressed were sequenced.

Bioinformatics

Each obtained sequence was edited to remove the adapter sequence. The edited sequences were used to query the GenBank database at NCBI using the BLAST sequence comparison algorithm for similar sequences. Selected sequences were also used to search EST database for *Solanaceae*.

Real-Time PCR Analysis

To confirm differential expression of SSH-based identified genes in transgenic plants vs the wild-type, the Real-Time PCR reactions were performed on full-length cDNA used for library construction. Than, for

expression analysis the RNA isolated from plants grown on medium with different Cd/Zn concentration was used. Analysis were performed in three biological repetitions.

RESULTS AND DISCUSSION

It was shown that the Cd concentration in plants used for the SSH-based analysis was reduced to 50%-75% of the concentration detected in WT (depending on the line). The shoot/root Cd concentration ratio in transgenic plants was at the WT level and the efficiency of cd translocation to shoot was not altered due to *AtHMA4*-expression.

To find out which molecular pathways leading to decreased Cd root and shoot level in *AtHMA4*-expressing tobacco were modified due to expression of transmembrane export protein AtHMA4, the transcriptional profiling of Cd-treated roots was analyzed using suppressive subtractive hybridization (SSH). Sequence analysis of 350 clones from each libraries was performed. Among others, we have identified genes that were differentially expressed in roots of transgenic plants which encode ion-transporters but also genes that control various developmental and physiological processes (amino-acid-, lipid- and carbohydrate metabolism, protein modification, ribosomal proteins, defense-related and stress-related proteins, and other).

There were selected 25 genes, encoding proteins involved in the following processes: Zn/Fe homeostasis, nitrogen metabolism, cell wall metabolism, pathogenesis-related, and osmotic-stress related. Their differential expression in transgenic plants in comparison to WT in both biological replications used for library construction was double-checked by Real-time.

Five genes participating in Zn/Fe homeostasis were selected for further expression analysis of plants exposed to a range Zn and Cd concentrations: *NtNAS1 and* four genes from the ZIP family.

Based on performed analysis the major altered pathways possibly contributing to the generation of low shoot Cd tobacco could be proposed.

ACKNOWLEDGEMENTS

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Biofortification of Zinc and Iron Grain Content of an Efficient Cadmium Excluding Winter Wheat Genotype by Non-Gm-Fast-Track Breeding and Mutant Selection Under Real Field Condition

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INTRODUCTION

In the past decades, both crop breeding and production has largely focused on maximising yield in terms of biomass production per hectare (green revolution) and on securing food and feed production. As a negative side effect of this strategy concentration of essential micro-nutrients for man and animals (e.g. Fe, Zn, Mg, Se, vitamin C) have been dramatically diluted. Estimates say, that over three billion people are currently micronutrient mal-nurished, resulting in high social costs including learning disabilities among children, increased morbidity and mortality rates, lower worker productivity, and high healthcare costs, all factors diminishing human potential, and national economic development. Nutrition deficiencies account for almost two-thirds of childhood death worldwide, and most of those afflicted depend on staple crops for their sustenance.

METHODS

The team of Phytotech Foundation (CH) introduced in 2011 a mutagenesis approach with the best Cd-excluding winter bread-wheat cultivar BATIS of Germany, obtained from Strube Research. The aim of this project is to biofortify the grain content of BATIS for desired Zn, Fe (and possibly Mg, Se) and exclude un-desired toxic elements such as Cd. Based on existing experiences with sunflower a modified EMS mutagenesis was used to produce a significant amount of mutants for direct field screening on our phytoremediated mainly zinc contaminated site of Bettwiesen (CH). About 20'000 mutagenized grains (0.08M and 3, 4, 7, 11 hours of EMS treatment) were sown in autumn 2011.

RESULTS AND DISCUSSION

First M_2-mutant freeland screening 2011/12

A few thousand of fertile mutant spikes and their controls were harvested at the Bettwiesen site (CH) in August 2012, and mutant screening was done in laboratory with grain of individual mutants and non-mutagenized controls on a well calibrated and sensitive XRF instrument. For the the more promising 11-h EMS-treatment M_1-mutants of 2012 reached a strongly enhanced variation with better accumlating traits. The relative grain gain of iron was up to a factor of 12.6, for zinc up to 4.7 (Fig.1a,b), as compared to controls, Fig.2a,b). For the cumulative Fe & Zn the grain biofortification was enhanced up to a factor 7.7 (Fig.3a,b). For the 7h EMS-treatment best M_1 mutants reached a relative gain of iron of 5.7 and for zinc up to a factor of 6.5, and for cumulative Zn & Fe biofortification was up to a factor of 4.

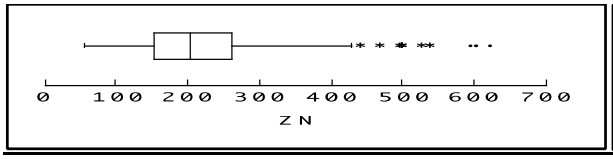

Fig. 1a Zinc grain content of M_1- mutants of BATIS (11h EMS-Treatment)

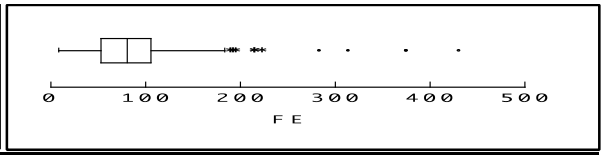

Fig. 1b Iron grain content of M_1-mutants of BATIS on Bettwiesen Soil

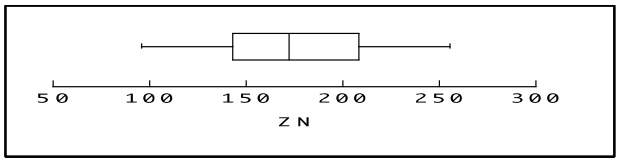

Fig. 2a Zinc grain content of BATIS Controls

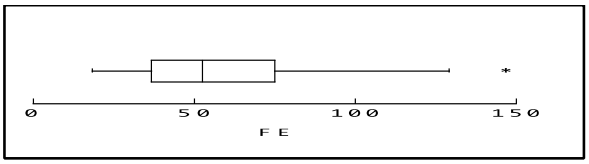

Fig. 2b Iron grain content of BATIS Controls

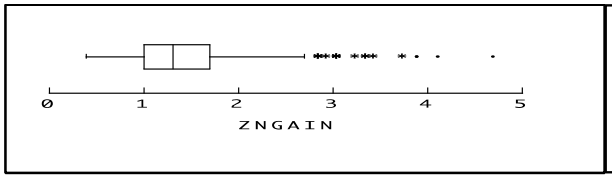

Fig. 3a Relative grain Zinc gain factor of BATIS M_1-mutants (11h EMS-treatm.)

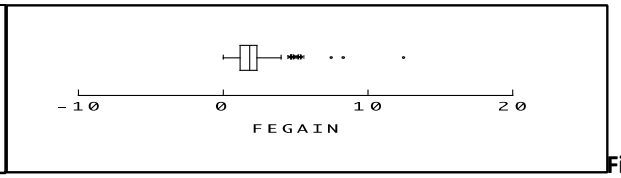

Fig. 3b Relative grain Iron gain factor of M_1-mutants compared to controls

Second mutant freeland screening of M_2 in 2012/13

Most promising individual M_1-mutants of 2012 with a cumulative gain factor of Fe & Zn > 1.9 were sown again in the Bettwiesen field (CH) under elevated zinc load of 500mg·kg^{-1} (HNO_3-extractable), and most promising inbred lines also in Söllingen (DE) on a soil with low zinc soil con-centration (60mg·kg^{-1}) for next M_2-generation in summer 2013. For the second mutant screening, we just analyzed about 3500 inbred-lines of the 11h-EMS treatment traits of Bettwiesen. We found 22 individual M_2-mutants with a cumulative Zn & Fe gain factor of the BATIS grain concentration > 3.0, whereas 7 individual mutants show a factor between 3.4 - 4. The best M_2-mutant show a cumultive Zn & Fe grain concentration of a factor 4.6, with a gain of 2.6 for Zn and 6.6 for Fe, as compared to non-mutagenized controls. For the most promising mutant lines we started the mutant screening on the low Zn & Fe contaminated German soil for clarifying, whether the biofortification is also inherent for “normal” Zn & Fe concentration on a healthy and productive agricultural soil.

CONCLUSIONS

The now available data of two subsequent field-based mutant screenings (M_1, M_2) at the Bettwiesen site (CH) on an elevated pseudo-total, but low labile zinc soil concentration show a remarkable biofortified Zn & Fe gain in grain concentration and stability of the selected mutant lines. We intend to continue the mutant screening with the most promising inbred lines to obtain more homogenous and biofortifed traits for intense investigations on the yield, pest and drought resistance, protein content and other plant based characteristics.

ACKNOWLEDGEMENTS

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Screening Wheat Germplasm for Zinc Uptake as Part of the WISP Programme in the UK

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INTRODUCTION

The WISP (Wheat Improvement Strategic Programme) project (formerly the Wheat Prebreeding LoLa programme), funded by the BBSRC in the UK comprises a consortium of five research teams (Rothamsted Research, John Innes Centre, Nottingham University, Bristol University and NIAB). The project aims to generate, genotype and phenotype novel wheat germplasm, with the focussed aims of increasing yields and resource use efficiency. As part of the programme selected germplasm is being screened for efficient mineral nutrient capture and partitioning between grain and straw at final harvest. The captured datasets include a range of micronutrients including zinc (Zn). As much of the germplasm has a low harvest index, the focus is on whole crop uptake rather than grain alone.

The germplasm, for which Zn uptake is reported here, consists of a subset of the Watkins Collection (Miller et al., 2001), a selection of the Gediflux collection (Reeves et al., 2004) and synthetic wheats provided by NIAB along with a number of modern germplasm controls. The Watkins collection consists of a genetically diverse collection of wheat land races collected from around the globe in the early part of the 20th century. This has been genotyped and a core collection is represented here. The strategy is to select individuals of interest and then screen derived mapping populations which have been created for the entire core set. Whilst only a single year/site data is shown in the abstract, the trials are being conducted at two UK sites and in multiple years.

METHODS

Field trials

The field experiment is a randomised block design, with three replicate blocks. Each block is equally split, one half receiving nitrogen fertilizer at a typical UK rate of 200kg N/ha, the other half receiving 50kg/ha. Only results from the high N plots are reported in this paper. The field experiment was sown 20th October 2011 and harvested on the 3rd August 2012 at Rothamsted Research in the UK. All plots received 200kg N/ha in three applications between 13th March and 22nd May. Dry matter grain and straw yields were measured for each plot, and sub samples kept for Zn analysis.

ICP analysis

0.025-0.05g of dried plant tissue samples were digested in 5 ml nitric acid:perchloric acid (85:15, v/v) (70% concentration, trace analysis grade, Fisher Scientific, Loughborough, UK), for a minimum of 2 hours at room temperature followed by 5 hour programmed thermoblock cycle. 5ml of 25% (v/v) nitric acid was added to the solution and the tubes were reheated for 1 hour at 80°C. Ultra-pure water (>18MΩ) was added to approximately 9ml (for experiments with smaller samples), this was mixed well and re-warmed for a further 30 minutes at 80°C. After cooling the solutions were made up to final volumes of 10ml with ultra-pure water. ICP-OES analysis was carried out using an Optima Inductively Coupled Plasma – Optical Emission Spectrometer (Perkin Elmer Life and Analytical Sciences, Shelton, USA).

RESULTS AND DISCUSSION

The Zn content and yields for both grain and straw were determined at harvest (data not shown). Combining these datasets allows the calculation of the total amount of Zn taken up. In Figure 1, this is shown as a function of the total biomass yield. The different germplasm origins are differentiated by the graphical symbol used.

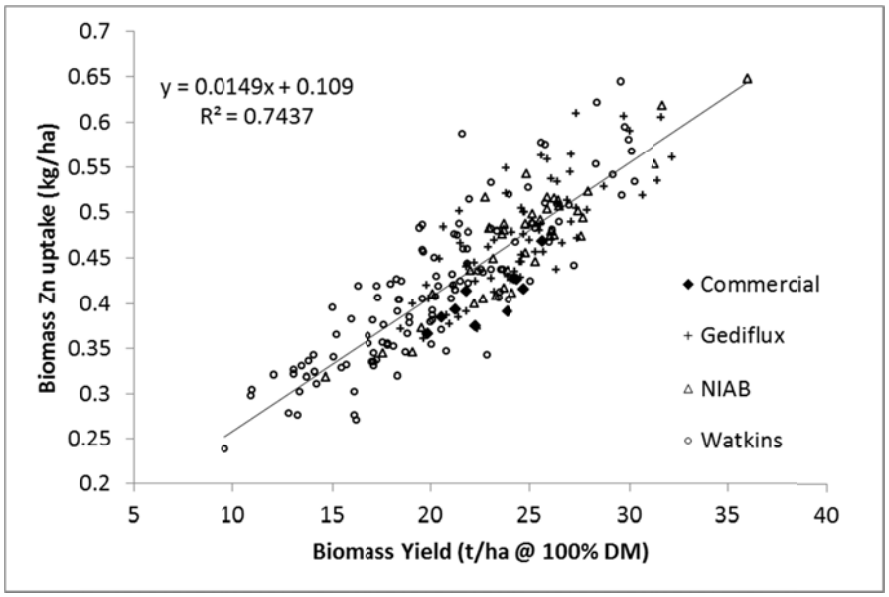

Fig. 1: Total biomass Zn uptake in a wheat field trial harvested in 2012.

CONCLUSIONS

Amongst the wheat germplasm surveyed there is variation in Zn content. The amount of Zn taken up by the crop is principally a function of biomass, the greater the biomass the more Zn is taken up. There are outliers with greater than expected Zn acquisition, notably amongst the Watkins collection, and these will be analysed further.

ACKNOWLEDGEMENTS

Rothamsted Research receives support from the Biotechnology and Biological Sciences Research Council (BBSRC) of the UK as part of the 20:20 Wheat® project. This project was funded as part of the BBSRC funded Wheat Prebreeding and WISP projects. NE receives funding as part of the BBSRC DTP scheme.

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How Metal Chelators Move Micronutrients - into Plant Organs and into the Human Body

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INTRODUCTION

Fe deficiency compromises both human health and plant productivity. In humans, Fe deficiency anemia is estimated to affect about 30 % of the world's population (WHO, 2002). Thus, it is important to understand plant Fe acquisition strategies for the development of crop plants which are more Fe-efficient under Fe-limited conditions such as alkaline soils, and have higher Fe density in their edible tissues.

Root secretion of phenolic compounds has long been hypothesized to be a component of the reduction strategy (strategy I) of Fe acquisition in non-graminaceous plants (Cesco et al., 2010). However, genetic evidence and specific molecular knowledge are lacking. Our objective was therefore to identify molecules involved in the acquisition of Fe by strategy I plants through a metabolomics approach. We subjected roots of *Arabidopsis thaliana* plants grown under Fe-replete and Fe-deplete conditions to comprehensive metabolome analysis by gas chromatography-mass spectrometry (GC-MS) and ultra-pressure liquid chromatography electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOF-MS).

METHODS

A. thaliana Col-0 wild type plants were cultivated hydroponically for easy access to root tissue. In order to allow stringent filtering of the highly complex metabolomics data, we imposed Fe deficiency conditions in two different ways in separate sets of experiments. First, four week old plants were grown for additional two weeks in medium without added Fe-HBED. Second, plants were cultivated for six weeks in medium with the pH adjusted to 7.7 (instead of 5.7). Roots were subjected to comprehensive metabolome analysis. The GC-MS setup consisted of a Gerstel MPS autosampler, an Agilent 7890A GC system and a 5975C inert MSD. A Waters Aquity UPLC system equipped with a HSS T3 column (1.8 µm, 2.1 x 100 mm) coupled to a Q-TOF Premier mass spectrometer was used for LC-MS metabolite profiling. To study the interaction of ferrous and ferric iron with coumarins, mixtures of ligands and Fe salts were analysed by direct infusion mass spectrometry and UV/Vis spectroscopy.

RESULTS AND DISCUSSION

GC-MS metabolite profiling of root extracts detected two dominant metabolic changes in plants grown in Fe-free medium relative to plants grown in control medium, namely increases in the abundance of the organic acids citrate and malate acid. This GC-MS profiling result validated our assay conditions.

Most of our analyses focused on LC-MS-based profiling which covers other compound classes than GC-MS and has the potential to provide structural information on unknown compounds. Scopoletin and other coumarins were found among the metabolites showing the strongest response to the two different Fe-limited conditions.

Biosynthesis of scopoletin has been molecularly elucidated (Kai et al., 2008) and the enzyme catalyzing a key step, the ortho-hydroxylation of cinnamates, is known as F6´H1. Respective coumarin biosynthesis mutants showed no obvious phenotypes when grown on normal soil. On alkaline soil, however, a dramatic effect on development was observed. The *f6´h1* mutants were unable to grow in the absence of Fe fertilization (Fig. 1). In hydroponic culture at alkaline pH, *f6´h1* mutants also showed impaired growth and displayed severe chlorosis.

Co-cultivation with wild-type plants partially rescued the Fe deficiency phenotype indicating a contribution of extracellular coumarins to Fe solubilization. Exudates of wild type and *f6´h1* mutant plants grown hydroponically were analyzed by UPLC-ESI-QTOF-MS. Indeed, scopoletin and other coumarins including esculetin were detected in root exudates of wild-type plants.

Thus, coumarins appear to be secreted by *A. thaliana* roots and could indeed be involved in the mobilization of extracellular insoluble Fe(III). We directly tested this hypothesis in feeding experiments with *f6'h1-1* mutant plants grown hydroponically at alkaline pH. Coumarins scopoletin and exculetin were found to partially rescue the growth defect of *f6´h1*.

Direct infusion mass spectrometry as well as UV/vis spectroscopy indicated that coumarins are acting both as reductants of Fe(III) and as ligands of Fe(II).

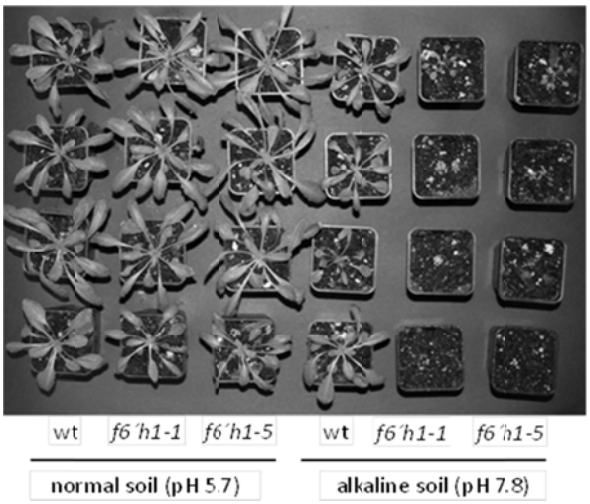

Fig. 1: Growth of coumarin-deficient *f6'h1* mutants is strongly impaired under Fe-limited alkaline conditions. *A. thaliana* Col-0 wild type plants (wt) and *f6'h1*T-DNA insertion mutants *f6´h1-1 and f6´h1-5* were grown on normal soil (left) and on soil alkalinized through the addition of CaO (right).

CONCLUSIONS

We demonstrate a crucial role of coumarin secretion for Fe acquisition by the strategy I plant *A. thaliana* under alkaline conditions. Coumarin biosynthesis in roots is enhanced under conditions of Fe deficiency. Coumarins are found in root exudates and contribute to Fe acquisition extracellulary. Our in vitro evidence suggests a Fe(III) reducing and Fe(II) complexing activity of coumarins as the underlying mechanism. Thesevfindings reveal a novel mechanism of Fe acquisition that may be used by a large number of plant species and has great potential for biofortification especially of crops grown under Fe limitation.

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Zn Biofortification in *Triticum aestivum* – A Clue on DNA Methylation

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INTRODUCTION

DNA methylation plays an important role in regulating gene expression during biological development and tissue differentiation. Environmental stresses can induce genetic and epigenetic changes that trigger DNA methylation, which can generate novel and heritable phenotypic variations. That could be the reason for the observed morphological changes in wheat spikes after successive generations of high levels of Zn application. Therefore, in this study we assess the extent and pattern of cytosine methylation in Zn biofortified *Triticum aestivum* L. *cv. Roxo* leaves.

METHODS

Parental (F0), F1 and F2 generation seeds of *Triticum aestivum* L. cv Roxo were sown in pots in a walk-in growth chamber, under environmental controlled conditions (80% RH; 24/20^{o}C day/night temperatures; PPFD of *ca.* 700 µmol m^{-2} s^{-1}, 12 h photoperiod). F0 and half of F1 seedlings were irrigated with a standard solution until harvest. F2 and half of F1 seedlings were irrigated with a standard solution (s) during 1 month after germination, and thereafter with 5 fold nutrients concentration (5s) until harvest. At the 91st day, DNA was extracted from leaves of each treatment, through homogenization in a Speedmill P12 (Analytik Jena). DNA was isolated using the Plant DNA Extraction Kit (Analytik-Jena) according to the manufacturer instructions. Methylation sensitive amplification polymorphism (MSAP) was done according to the protocol developed by Reyna-Lopez *et al.* (1997). PCR products were co-loaded with a GeneScan 500 ROX size standard into an ABI Prism 310 Genetic Analyzer (Applied Biosystems). Fragment analysis and AFLP scoring was performed using Genescan 3.2 software (Applied Biosystems) and scored manually for the presence (1) or absence (0) of polymorphic bands across genotypes. MSAP data for all primer combinations were mixed for each plant and the samples were organized into four populations considering the Zn supplementation treatment: F0 (s), F1 (s), F1 (5s), F2 (5s). Statistical analysis of MSAP results was performed using the Statistica and JMP software. Epigenetic differentiation based on Nei's genetic distance was assessed with principal components analysis (PCA) performed in GENALEX 6.4.

RESULTS AND DISCUSSION

DNA methylation at CCGG sites was analyzed using 35 pairs of selective primers, obtained from five EcoRI primers in combination with seven fluorescently labeled HpaII/MspI primers. The individual DNA methylation profiles was generated by F-MSAP. Each individual genome with each primer combination was analyzed with primer combination after capillary electrophoresis using Genescan 3.2 software. A total of 859 fragments were detected in DNA extracted from wheat plants (F0, F1 and F2 progeny) subjected to Zn biofortification treatments. For each primer combination, each individual genome displayed 2-20 fragments. In particular, fragments between 100 bp and 500 bp were highly intense. Each of the 859 Amplicons represented a recognition site cleaved by either or both *Eco*RI + *Hpa*II and *Eco*RI + *Msp*I. Still, 149 (17%) fragments were produced from cleavage by *Msp*I but not *Hpa*II; 440 fragments (51%) resulted from cleavage by *Hpa*II but not *Msp*I; 268 fragments (31%) arose from cleavage by both restriction enzymes. For the analysis three types of methylation status were considered: type I for

amplicons of the same length present in both HpaII and MspI electropherograms, which indicates inner methylation of single-stranded DNA or no methylation; type II for amplicons present for HpaII but absent for *Msp*I digests, which indicates outer methylation of single-stranded DNA and hemi-methylation at the outer cytosine nucleotide in the CCGG sequence; and type III for amplicons present for MspI but absent for *Hpa*II digests, which indicates inner methylation of double-stranded DNA and full methylation of the CCGG sequence. At a percentage basis type II was the most frequently observed, followed by type I and type III, respectively for each treatment (Table 1). The sum of types II and III represents all the methylated fragments.

	Methylation type			
Plant treatment	**Type I**	**Type II**	**Type III**	**Total methylation %**
F0 (s)	25.00	53.30	21.70	75.00
F1 (s)	32.00	48.00	20.00	68.00
F1 (5s)	36.65	50.68	12.67	63.00
F2 (5s)	31.13	51.99	16.56	68.54

Table 1. Methylation type (%) on the methylation status

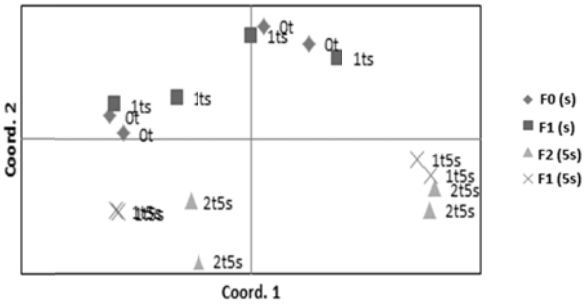

Fig. 1. Principal Coordinates Analysis (PCoA) based on Nei's epigenetic distance.

According to the obtained data the increase of Zn concentration causes a decrease in the percentage of total methylated cytosines (sum of types II and III). Particularly, type III methylation status which indicates inner methylation of double-stranded DNA and full methylation of the CCGG sequence seems to be affected by the (5s) biofortification treatment. Data from F0 (s) to F1 (s) plants, show a slightly difference between type III percentages (21.70 and 20.00) that was displaced to types I and II status. This might be due to genetic changes between F0 and F1 plants. For the (5s) treatment, a coincident decrease of the percentage of type III methylation was found, compensated with the increase in type I status methylation, which indicates inner methylation of single-stranded DNA or no methylation. Accordingly, data suggest that the (5s) treatment induces a demethylation process in the leaf DNA of wheat plants treated with Zn (Table 1). The results showed that there are different methylation levels and patterns under different conditions, and that the differences are significant ($P \leq 0.001$). PCA analysis, considering polymorphic bands from the MSAP technique, further shows different clustering of samples (Fig. 1). The coordinate 1 is explained by the different band profiles produced by the methylation sensitive digestion of *Hpa*II and *Msp*I. However, coordinate 2 divides samples under nutrient solution treatment clustering samples under (s and 5s) treatment samples in the upper and lower part of the plot, respectively. This clustering may be explained by the differential methylation status resultant from the two applied concentrations of nutrient solution ("s" and "5s").

CONCLUSIONS

A clue for DNA demethylation mechanism response to Zn biofortification was obtained, indicating that the differences in the methylation levels and patterns are probably implicated in the regulation of specific gene expression and cell differentiation. Further studies should clarify this issue.

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Natural Variation of Magnesium Content in *Arabidopsis*

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INTRODUCTION

Magnesium (Mg) is the fourth-most common cation in the human body and half of its dietary intake is from plant origin (reviewed in Hermans et *al*. 2013). Worldwide, nearly two-thirds of the population does not consume the recommended amount of Mg in their diet. Therefore, understanding how plants take up Mg from soil and regulate their internal content could have significant implications for offering humans improved Mg sources to help overcome malnutrition. Our primary interest is to gain better knowledge on the mechansims governing Mg homeostasis in the model species *Arabidopsis thaliana*, a domain that is relatively unexplored. To achieve this goal, our experimental outline is to exploit changes in tissue mineral concentrations of natural Arabidopsis populations and to identify genes and alleles controlling Mg homeostasis. Since those populations grow in contrasted soil conditions, genetic variation is expected to be found in traits linked to mineral uptake and tissue concentration. We observed an 50% difference variation in Mg concentration of root and leaf organs between the most contrasted accessions of the 96 Nordborg collection grown in hydroponics (Baxter et *al*., 2012) and 351 HapMap grown on soil (www.ionomicshub.org) (Fig. 1).

METHODS

Our aim is to identify loci controlling the elemental concentration in plants through linkage mapping, and genome-wide association mapping strategies. We detail here the first approach. QTL analyses are carried with the progeny between contrasted accessions for which recombinant inbred lines (RILs) are available in seed stock centres. Two RIL families were screened upon hydropnics culture: about 160 lines generated from the cross between the reference Columbia (Col-0) and Yosemite (Yo) identified as low Mg (-13%), or Col-0 and Shadara (Sha) identified as high Mg (+13%) (Fig. 1B, Fig. 2).

RESULTS AND DISCUSSION

QTL analyses releaved more than a dozen of chromosomic intervals with LOD scores between 2.5-10 and explaining 10-25% of the phenotypic variance observed for the elemental profile in both Sha and Yo-0 mapping populations. Some of those QTLs co-locolize with known ion permeases, such as *MOLYBDATE TRANSPORTER 1* in the case of molybdenum (Fig. 3B). Only one QTL with a LOD score just above threshold was detected for the leaf Mg concentration of the Col-0 x Yo-0 couple (Fig. 3A).

CONCLUSION

Drawback to detect Mg QTL can be attribuated to the limited choice of publically available RIL families (nearly 13% difference between the parent accessions tested here). Fort that reason, we are currently creating new experimental mapping populations issued from the cross between the most contrasted accessions grown on soil: high Mg (Sg-1, Ull2-5, Ws, Fja2-1, Ste-0) X low Mg (PA-5, Cvi, PHW-36, Ors-2, Tamm-2) (Fig. 1C). A more promising approach would be the deep sequencing of DNA pools of F2 individuals issued from those crosses.

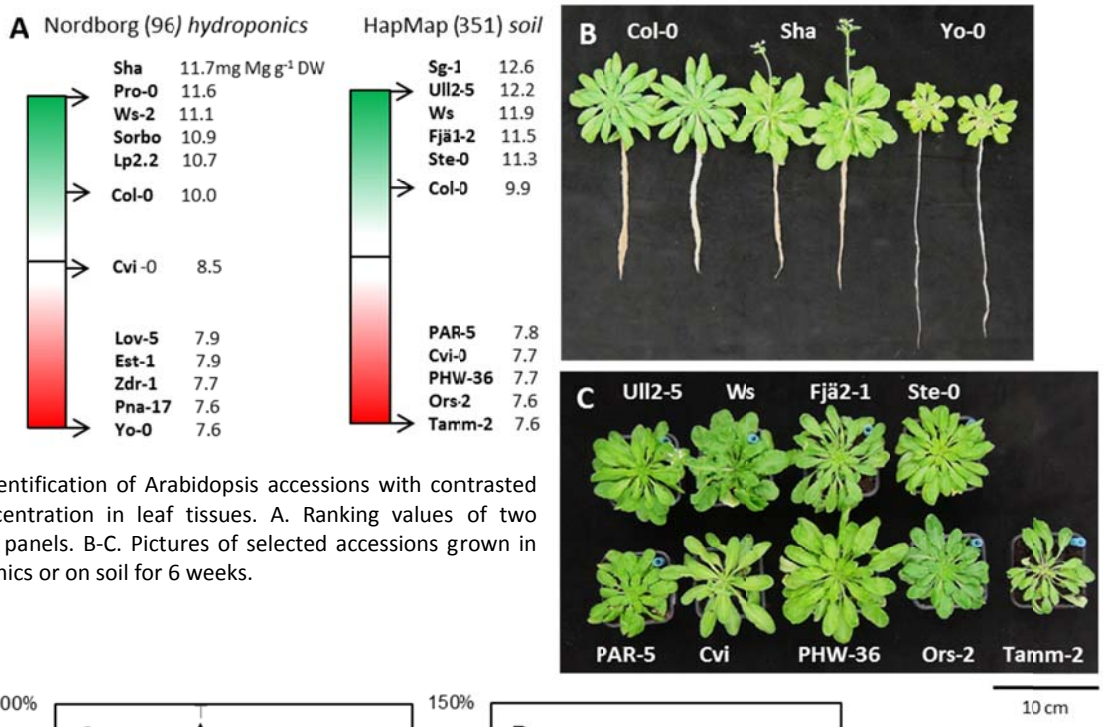

Fig. 1 Identification of Arabidopsis accessions with contrasted Mg concentration in leaf tissues. A. Ranking values of two diversity panels. B-C. Pictures of selected accessions grown in hydroponics or on soil for 6 weeks.

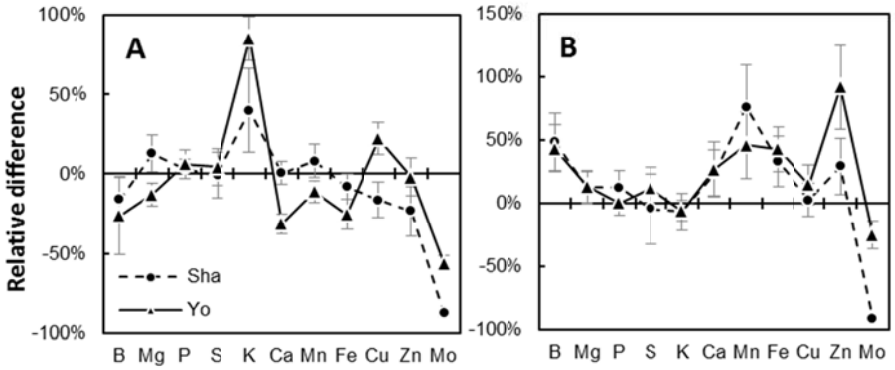

Fig. 2 Ionomic profiles of parent accessions used for QTL mapping. Values are expressed relatively to Col-0, in young mature leaves (A) and roots (B) upon hydroponics culture. n≥ 20 ± std

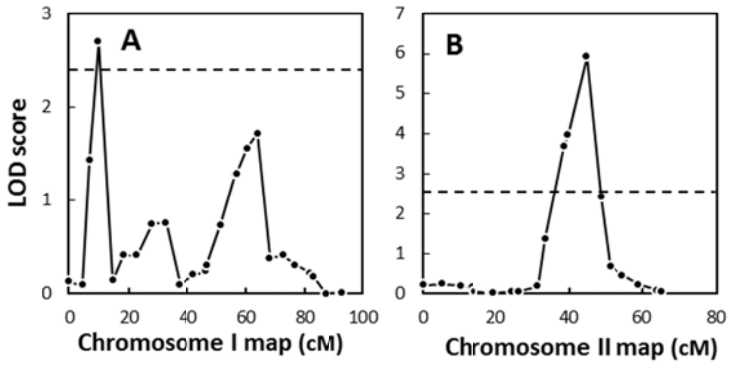

Fig. 3 Selected LOD profiles for Mg (A) and Mo (B) leaf concentration of 160 RILs (Col-0 x Yo) grown in hydroponics. The QTL treshold is shown by the dashed line.

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Natural Variation for the Response to Zn Deficiency in *Arabidopsis thaliana*

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INTRODUCTION

Plants need zinc (Zn) as an essential micronutrient for many cellular processes, and like all other organisms, they evolved a regulatory system to control the plant and cellular Zn status, ensuring Zn homeostasis. Previously we cloned two transcription factor (TF) genes from *Arabidopsis thaliana*, *bZIP19* and *bZIP23*, which act redundantly in controlling the initial Zn deficiency response (Assunção et al., 2010). We are interested to know how these TFs act in controlling the Zn deficiency response, i.e. which are their transcriptional targets and partners and how do they receive the Zn deficiency signal (Assuncao et al., 2013). Although these TFs are essential for plants, there are likely to be additional regulators of the adaptation to low Zn supply. We are interested to identify these and study natural genetic variation for the response to Zn deficiency in order to do so. We used RNA-Seq and Genome Wide Association (GWA) mapping to obtain further genetic clues on the regulation of Zn homeostasis in *A. thaliana*.

RESULTS

In a screen for natural genetic variation for response to Zn deficiency, we examined a collection of ~350 natural *A. thaliana* accessions and identified some 20 diverse accessions showing different physiological responses, including differences in Zn deficiency induced gene expression. Two accessions with extreme and contrasting phenotypes for Zn deficiency tolerance, and the reference accession Col, have been used for RNA-Seq transcriptome analysis. This identified several genes to be either involved in the *A. thaliana* core response to Zn deficiency, or in the accession specific Zn deficiency response. The same screen, in combination with elemental analysis of accessions, has been used for a Genome Wide Association Study. This revealed many candidate loci involved in the (adaptive?) response to Zn deficiency.

DISCUSSION

Although this work has not been finished, with several candidates found, but few verified, this kind of research is expected to expand our knowledge on ecologically, and possibly also agronomically, relevant genetic factors controlling micronutrient homeostasis and also to identify new genes, beyond the range of mineral transporter genes that are already known to be involved. In addition, it will be interesting to compare natural variation for Zn homeostasis in *A. thaliana* with the natural genetic variation recently investigated in rice (Norton et al., 2014) or the Zn/Cd hyperaccumulator species *Noccaea caerulescens* (Halimaa et al., 2014), to identify common factors that will be interesting for further gene function studies.

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Session-III:
Product Processing and Human Nutrition

Sustainable Diets: Implications of Climate Change for Nutrition and Health

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The current food system and consumption patterns worldwide are unsustainable both for population health and the environment. Nutritional problems are increasing throughout the world with under-nutrition (e.g. hunger, nutrient deficiencies) and over-nutrition (e.g. obesity and related chronic diseases) often coexisting in the same country. What we eat, however, not only impacts directly on our own health but also on the environment and climate change, which impacts indirectly on the health and lives of others. Raised awareness of climate change and concern about future food security has renewed an interest in sustainable diets. The concept of sustainable diets is not new; in 1986 Gussow and Clancy proposed that dietary guidelines should take into account wider issues such as the environmental impact of dietary choices, and then later in 2005 The Giessen Declaration called for food and nutrition policy to include environmental sustainability, recognizing that the food system is contributing to environmental damage (e.g. production of greenhouse gas emissions (GHGE), land use change, increasing water usage, loss of biodiversity, degradation of ecosystems and increasing pollution).

While dietary recommendations have primarily been concerned with health outcomes, they should be revised to include wider environmental and social issues around food choices. Some countries, such as the Netherlands and Sweden, have started to address this issue and develop guidelines for healthy sustainable diets. This adds a layer of complexity, not least because the recommendations are not always compatible; many dietary guidelines recommend minimum intakes of fish, but from an environmental perspective this conflicts with concern over limited natural fish stocks and the environmental impact of farmed fish. These types of issues need to be resolved to ensure the different elements of sustainability (e.g. health, environment, social, economic) are linked together and thereby avoid unintended consequences, which may occur if they are considered in isolation.

What are sustainable diets? One of the few definitions of sustainable diets that exist was produced at an international symposium hosted by the Food and Agriculture Organization in 2010. This definition (below) highlights the complexity in bringing together all the aspects of sustainability for truly sustainable diets.

Sustainable Diets are those diets with low environmental impacts which contribute to food and nutrition security and to healthy life for present and future generations. Sustainable diets are protective and respectful of biodiversity and ecosystems, culturally acceptable, accessible, economically fair and affordable; nutritionally adequate, safe and healthy; while optimizing natural and human resources (Burlingame & Dernini 2010).

Sustainable diets are important for future food security. Looking to the future and bringing together the need to alleviate poverty, predicted population growth, increasing urbanisation, reducing availability of finite resources and climate change has been described in relation to food as the 'perfect storm' (Beddington 2009), and highlights the need for change. The current food system, for example, is contributing significantly to global climate change and it is estimated that it accounts for between 20-30% of total GHGE. GHGE are produced through the whole food system from agriculture through processing, transportation, retail, consumption and waste, with agriculture and food production accounting for the majority of emissions. Emissions vary by food group (and production methods) with animal based products, especially from ruminant animals, having higher GHGE than most plant based products. High GHGE of meat based diets combined with increasing meat consumption across the world has lea to suggestions that consumption of meat should be reduced. It is important here to remind ourselves of the definition of sustainable diets and ensure that any dietary changes proposed to limit

the environment damage consider any nutritional consequences. Initial studies have illustrated that it is possible to create diets that can meet dietary requirements for health and reduce GHGE, but it should not be assumed that a healthy diet will always be lower in GHGE (Macdiarmid *et al.* 2012). More work, however, is required to incorporate other environmental, social and economic factors to fully understand how sustainable diets.

While the food system is contributing to climate change, in turn climate change will determine the types of food and crops that can be produced in different parts of the worlds, with implications for diet and health. Changes in the food system are needed to improve the health of the population and limit the environmental damage, but any changes have to be culturally, socially and economically sensitive to the requirements of different populations. The challenge to change production methods and consumption patterns is not underestimated because of the many competing priorities that exist. Debates are on-going as to whether the focus should be on production or consumption; should we focus on reducing methane production in cows through the use of feeds and agricultural methods or should we focus on eating less meat? In reality both have to be considered options because as many years of nutrition and public health have illustrated there is no single solution to these complex issues. Furthermore we need to move away from a reductionist approach to nutritional and dietary problems in order to understand the wider consequences in order to move towards more sustainable diets in practice.

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Efficacy of Consuming Iron-Biofortified Staple Food Crops

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INTRODUCTION

After plant breeders have successfully developed varieties of selected staple foods to increase the iron content, the edible portion of the food crop must be tested for a number of qualities before they can be considered for introduction to the food supply. Efficacy, or the demonstration of a significant impact on the nutritional status of human subjects who consume the staple food under controlled experimental conditions, must be demonstrated. To date three iron-biofortified staple foods, beans (in Mexico and Rwanda), rice (in the Philippines) and pearl millet (in India) have been tested for efficacy in populations that consume the food as a major component of their normal diet. A systematic evaluation of all of the iron-biofortification efficacy studies will be presented.

METHOD

All 4 efficacy studies employed a randomized, controlled, experimental study design. The iron-biofortified or similar control food was prepared to local tastes. Human research subjects consumed the foods for between 100 and 270 days, depending on the study, and meal consumption was monitored that allowed observation of dietary iron intakes from the total diet as well as precise quantification of the iron consumed from the staple food of interest. All studies assessed iron status (blood hemoglobin, serum ferritin, soluble transferrin receptor and total body iron) at baseline and again at the end of the feeding trail and analysis focused on the feeding group difference in the change in iron status between baseline and endline.

RESULTS AND DISCUSSION

The first efficacy study demonstrated “proof-of-concept” when consumption of iron-biofortified rice for 9 months resulted in an increase in serum ferritin and total body iron in non-anemic Filipina religious sisters (Table 1). Biofortified pearl millet was evaluated in secondary school children from western Maharashtra, India. A significant improvement in serum ferritin and total body iron was observed in iron deficient adolescent boys and girls after consuming pearl millet-flat bread twice daily for 4 months. Biofortified beans were tested for efficacy in two very different populations. Mexican primary school children were observed to have improved transferrin receptor levels after consuming biofortified black beans for 105 days; however, the acute phase protein, serum ferritin, did not improve, primarily because of high levels of infection in this population. Iron deficient Rwandan university women showed a significant increase in hemoglobin and total body iron after consuming biofortified beans for 6 months.

Iron concentration in the consumed portion of the staple food was lowest in rice (9.8 mg/kg) compared to the other 3 crops (86-97 mg/kg). Absorbable iron from the biofortified food ranged from 134 mg/d for rice to 1300 mg/d for pearl millet, with most of the variation related to the quantities of the staple food consumed daily (Table 2)

The strength of the findings for each study and across all four studies will be evaluated relative to the iron status of the study population at baseline, their inflammation status, dietary components that might affect absorption, dose of absorbable iron consumed, subject compliance and length of feeding time. These results will be discussed in terms of how to improve future efficacy studies for iron biofortified crops.

Table 1: Comparison of Results from Four Iron-Biofortification Efficacy Studies

Staple Food Crop (location)	Rice (Philippines)		Beans (Rwanda)		Beans (Mexico)		Pearl Millet (India)	
Subjects	Adult Females		Adult Females		Children (M+F)		Youth (M+F)	
Experimental group	High Iron	Control	High Iron	Control	High Iron	Control	High Iron	Control
Number of subjects	69	69	116	118	269	166	99	98
Hemoglobin (g/dL)	0.11	0.09	*0.3* [a]	*-0.10*	0.00	0.60	-0.14	-0.15
Ferritin (µg/L)	*1.1* [a]	*-4.27*	4.04	2.65	3.20	5.20	*5.7* [a]	*1.2*
Transferrin receptor	0.35	-0.15	-0.26	0.09	*-0.10* [a]	*0.10*	*0.19*	*0.21*
Body iron (mg/kg)	*0.63* [a]	*-0.25*	*1.36* [a]	*0.43*			*0.83* [a]	*0.02*
Sample Description	Non-anemic (Hb>12g/dL) at baseline		Low ferritin (<20 µg/L) at baseline		Low morbidity-inflammation schools		Low ferritin (<15µg/L) at baseline	

[a] Significant difference between high and low iron groups, Wilcoxon 2-group comparison test, p<0.05

Table 2: Iron Intakes from Biofortified Staple Food

Staple Food Crop	Rice		Beans (Rw)		Beans (Mex)		Pearl Millet	
Experimental group	High Iron	Control	High Iron	Control	High Iron	Control	High Iron	Control
Iron content								
Concentration (µg/kg-dry)	9.8	1.9	86	51	95	55	87	30
Intake from staple (mg/d)	1.8	0.4	13.5	8.0	4.7	2.6	17.6	5.7
Percent of total dietary iron	18	5	64	46	26	19	90	81
Iron intake relative to requirements								
Percent iron absorption[a]	7.3	7.3	7.3	9.2	5.0	5.0	7.4	7.5
Absorbable iron (µg/d)	134	30	986	737	233	132	1300	428
EAR for iron (µg/d) [b]	1460		1460		800		1060	
Percent EAR from staple	9	2	68	51	29	17	123	40

[a] Iron absorption estimates: Philippines rice from by Beard et al (J Nutr 137:1741;2007); Rwanda beans from Petri et al (*J Nutr 143:1219;* 2013); Mexico beans estimated from Halberg & Hylthen (*AJCN* 71:1147;2000); Pearl millet from Cercamondi et al (*J Nutr* 143:1376; 2013)

[b] EAR = Estimated Average Requirement (from IOM, *Dietary Reference Intakes-DRI* ; 2001

CONCLUSION

Iron biofortification of select staple food crops has been shown to be efficacious when feeding trials followed specified guidelines to ensure: a) adequate iron difference exists between high iron and control groups, b) subjects were iron deficient at baseline, c) sufficient consumption of the staple food was documented, d) adequate time elapsed to see a response, and e) appropriate biomarkers of iron status were used. *Supported by HarvestPlus.*

Zn and Fe Biofortification in *Triticum aestivum* – Nutritional Evaluation of the Grain

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INTRODUCTION

Zinc and Fe biofortification of staple foods, namely *Triticum aestivum* crops, might overcome these nutrients deficiencies in humans. Yet, following a metabolic perspective, it might also change the patterns of fatty acids, carbohydrates and proteins, which therefore can alter the nourishing value of the flour. In this context, this work aims at assessing potential implications, in environmental controlled conditions, of bread wheat biofortification on these parameters.

METHODS

Parental (F0), F1, F2 and F3 generation seeds of *Triticum aestivum* L. cv Roxo were sown in a walk-in growth chamber, under environmental controlled conditions (80% RH; 24/20^{o}C day/night temperatures; PPFD of *ca*. 700 µmol m^{-2} s^{-1}, 12 h photoperiod). F0 (s), F1 (s), F2 (s), F3 (s/s) and F3 (7s/s, 10s/s) were irrigated with a standard solution until harvest. F1 (5s, 7s, 10s) and F2 (5s, 7s, 10s) pot seeds were irrigated with a standard solution (s) during 1 month after germination, and thereafter with 5, 7 and 10 fold nutrients concentrations until harvest. The seeds: F3 (s/s, s/5s) were obteined from F2 (s) and F3 (s/s) plants grown with standard solutions, respectively; F3 (5s/s, 5s/5s) were obtained from F2 plants grown with 5 fold higher concentrations of all micro and macronutrients, respectively. F3 (s/5s) and F3 (5s/5s) pot seeds were irrigated with a standard solution (s) during 1 month after germination, and thereafter with 5 fold nutrients concentrations until harvest. Scanning electron microscopy (SEM) studies were performed on wheat grains, using a Jeol 330. The lipid fraction of the seeds was extracted in a mixture of chloroform/methanol/water (1:1:1, v/v/v). After evaporation of the chloroform layer, the dry residue was resuspended in 1 cm^3 of a mixture of ethanol/toluene (1:4, v/v) and stored at -20 °C until analysis. After saponification with 0.5 M NaOH in methanol, the fatty acids of lipid extracts were methylated with BF3 and analyzed by gas liquid chromatography (UNICAM 610 gas chromatograph, Unicam, Cambridge, UK). Analysis of carbohydrates were carried out on ground samples ($\leq$ 0.5 mm sieve): mono and disaccharides by HPLC (Waters system, equipped with a SugarPakI column and a 2414 RI detector, both from Waters); starch by polarimetry. Ash was determined after seeds calcination at 550^{0}C for two hours. Total protein was determined following the Lowry protein assay.

RESULTS AND DISCUSSION

Supplementing wheat plants with all macro and micronutrients proportionaly, between 2 sucessive generations, there was a significant increase on the amount of nutrients in bread wheat grains. Yet, from the 3rd generation onwards, if adequate or 5 times higher amounts of all nutrients are provided in the nutrients solution, a plateau is achieved for Fe and Zn contents in the grain (data not shown). In this context, total fatty acids (TFA) had maximum values in F2 (5s, 7s), yet did not vary significantly between both generations and among treatments (with the exception F2 10s) (Table 1). Concerning individual fatty acids (Table 1), despite of some minor variations, it was found that C16:0 remained consistently higher in the biofortified crops, which can be benefitial from a nutritional and therapeutic point of view, since palmitic acid is an “energy storage” molecule, having an important structural role in cell

membranes (Leshem, 1992) and also seems to ensure mild antioxidant and antiatherosclerotic properties (French *et al*., 2002). Besides, the potential yield of crop production in the field, is only possible with F2 (s and 5s) (data not shown), it is important to note that that in plants supplemented with lower concentrations of macro and micronutrients (*i.e.*, F3 5s/s, relatively to F3 5s/5s), the relative amount of FAs do not decrease (Table 1). As regards DBI, lower values (lower degree of unsaturation) were observed, relatively to F0 (s) in F2 grains (Table 1). As regards carbohydrates, higher values were found for raffinose, sucrose, glucose and fructose in F2 (s) and F2 (5s) in relation to F0 (s), whereas starch decreased significantly from F2 (5s) onwards (Table 1; Fig.1). Between F0 (s) and F2 protein and ash levels progressively increased, with F3 further displaying a similar pattern (Table 1).

Table 1. Nutritional parameters of biofortified crops of *Triticum aestivum* L.cv Roxo.

	***Fatty Acids** (mol %) ± S.E.*					***TFA***	***DBI***
	C16:0	**C18:0**	**C18:1*c+t***	**C18:2**	**C18:3**	**(mg/g dw)**	
F0 (s)	18.5±0.3	1.0±0.0	15.1±0.3	59.5±1.1	4.5±0.1	6.8±0.1	7.0±0.2
F2 (s)	24.14±0,8	1.02±0.1	12.29±0.2	53.37±0.7	3.50±0.1	7.9±0.2	4.5±0.0
F2 (5s)	22.40±1.2	0.73±0.1	13.10±0.4	52.73±0.8	4.68±0.1	8.6±0.3	4.9±0.0
F2 (7s)	19.19±0.2	1.28±0.1	12.20±0.1	50.98±0.5	5.75±0.2	8.6±0.1	4.8±0.0
F2 (10s)	19.81±0.6	1.44±0.2	12.28±0.1	50.98±0.7	5.93±0.1	4.4±0.0	4.8±0.0
F3 (s/s)	18.8±0.3	0.7±0.1	14.7±0.2	59.5±0.3	4.8±0.3	6.7±0.3	7.1±0.1
F3 (5s/s)	19.7±0.2	0.8±0.0	14.3±0.1	61.2±0.1	4.7±0.0	6.9±0.2	6.8±0.1
F3 (5s/5s)	20.0±0.1	0.9±0.0	15.1±0.0	59.8±0.1	4.9±0.0	6.7±0.2	6.6±0.0
	Carbohydrates, Protein and Ash (%) ± S.E.						
	Raffinose	***Sucrose***	***Glucose***	***Frutose***	***Starch***	***Protein***	***Ash***
F0 (s)	1.02±0.01	1.73±0.01	0.15±0.01	0.06±0.00	38.424	10.5±0.6	1.70±0.16
F2 (s)	1.07±0.01	2.23±0.01	0.48±0.01	0.19±0.01	39.840	20.1±0.7	3.09±0.17
F2 (5s)	1.03±0.02	1.91±0.02	0.41±0.01	0.22±0.00	30.239	32.1±0.9	3.61±0.93
F2 (7s)	0.71±0.00	1.32±0.02	1.27±0.00	0.92±0.04	21.205	33.2±1.8	4.10±0.68
F2 (10s)	0.48±0.05	0.77±0.17	1.21±0.01	0.88±0.02	18.571	36.5±1.2	4.55±0.08
F3 (s/s)	1.21±0.10	1.44±0.10	0.02±0.01	0.03±0.01		18.2±1.1	3.02+0.15
F3 (5s/s)	1.36±0.08	1.52±0.08	0.01±0.00	0.03±0.00		24.1±0.9	3.01+0.20
F3 (5s/5s)	1.06±0.12	1.63±0.17	0.01±0.00	0.03±0.00		28.2±2.0	3.67+0.89

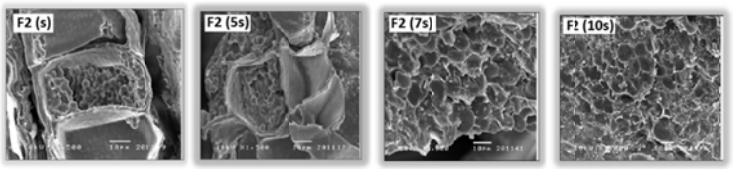

Fig. 1. SEM of starch in seed cells, showing a sharp desorganization in F2 (7s, 10s), which are grains without potential for field production.

CONCLUSIONS

Zn and Fe biofortification of wheat crops are linked to nutritional changes due to variations on the patterns of fatty acids, carbohydrates, proteins and ash. These changes can not be attributed to environmental stresses as the plants grew under similar conditions. Moreover, it is not expectable that these nourishing parameters might limit flour consumption by human populations.

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Selenium Speciation in Biofortified Crops under Semiarid Conditions

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INTRODUCTION

Selenium (Se) is an essential dietary nutrient for humans and animals, but intake of Se is inadequate in more than 15-20% of the population. Food is the main route of Se intake and agronomic biofortification has shown to be the most effective way to increase Se content in edible crops. But not only the total Se intake is important, the chemical Se species formed is also important in the bioavailability. The US Institute of Medicine (2000) has reported that more than 90% of selenomethionine (Se-Met) is absorbed; selenocysteine appears to be very well absorbed. Almost 100% of selenate is absorbed, a significant fraction is lost in the urine; and although a 50% of selenite is absorbed, it is more retained than selenate in the organism. It is also known that Se-Met is the major Se species contained in the grain of many cereals and legumes (Hart et al., 2011). The aim of this research is study how different foliar Se aplications affect to such speciation in durum wheat and chickpea grain under Mediterranean conditions.

METHODS

The study was conducted in 2011/2012, in Badajoz, southern Spain (38º54' N, 6º44' W, 186 m above sea level), in a Xerofluvents soil under rainfed Mediterranean conditions. Two crops were studied; chickpea (*Cicer arietinum* L.) and durum wheat (*Triticum durum* L.). Each experiment was designed as a split plot arrangement with four repetitions, including each Se form (sodium selenate (Na_2SeO_4) and sodium selenite (Na_2SeO_3)) in each plot, and within the plot, four application rates (0-10-20-40 g ha^{-1} of diluted in 3 L of water) were randomly distributed. Fertilizers were applied at the end of tillering EC-39 in hard wheat and at flowering in chickpea, on sunny days as foliar application. Total Se in grain were determined using an Inductively-Coupled Plasma Mass Spectrometer (ICP-MS) operating in the hydrogen gas mode. To quantify the Se species, the four replicates of each treatment were bulked. Speciation was carried out by HPLC-ICP-MS following proteolytic digestion with protease and lipase using an Agilent 1050 HPLC pump and a Hamilton PRP-X100 250 x 4 mm column (Cole-Parmer, London, UK). Three replicates from the 10 g ha^{-1} of sodium selenate treatment were analyzed to study the technical repeatability. All the data were subjected to ANOVA analysis.

RESULTS AND DISCUSSION

Total Se concentrations in the non-treated grains were much higher in chickpea than in hard wheat. Speciation was not possible in the non-treated samples in hard wheat, due to the low total Se concentration. The extraction efficiency from the total Se following proteolytic digestion method varied with regard to the crop and treatment. The greater efficiency in the recovery of Se was higher in chickpea (71%) than in hard wheat (57%) (Table 1).

The proportion of Se-Met (51-98%) was in agreement with the speciation of Se in several crops like wheat or lentils, in which Se-Met was also the main component, ranging between 56-83% (Hart et al., 2011; Whanger, 2002). Although chickpea accumulated significantly more total Se in the grain (on average 1037 *vs.* 482 µg kg^{-1} DW), the proportion of Se-Met, the more bioavailable Se form for humans, was much higher in hard wheat than in chickpea (92 *vs*. 74%). Legumes could be able to accumulate a greater amount of total Se in the grain than cereals, and this could be due to the higher protein content in legume grain in comparison to cereal grain but its bioavailability is lower than in cereals. Sodium selenate, regardless of the crop and application rate, was much more efficient in boosting Se concentrations in grain than sodium selenite (on average 1208 and 508 µg kg^{-1} DW, respectively). The concentration of Se-Met was, in both crops, higher applying Se as sodium selenate compared to sodium

selenite (90*vs*. 79.5 %) (Table 1). It was expected that around 15% of the organic Se would be present in other forms, according to Hart et al. (2011) but only Se-Met was observed. Perhaps these other forms of Se organic remained in the proportion of Se not recovered by HPLC.

Table 1. Total Se obtained by ICP in samples from each treatment and Se recovered (expressed as %) in the HPLC for chickpea and hard wheat grains. Percentage of each Se species obtained from the relative peak areas (by HPLC) for each fertilization treatment. nd = not detectable.

Crop	Treatment		Total Se ICP ($\mu g\ kg^{-1}$)	Se recovered (%)	Selenium species (% of total Se)		
	Product	Dose			SeMet	Selenite	Selenate
Chickpea	Sodium selenite	0 g ha^{-1}	269.5	89.2	50.6	39.8	9.6
		10 g ha^{-1}	660.4	83.7	63.2	29.6	7.2
		20 g ha^{-1}	845.2	74.1	74.0	20.7	5.3
		40 g ha^{-1}	703.7	70.0	70.6	26.5	2.9
	Sodium selenate	10 g ha^{-1}	805.6	58.6	84.8	8.5	6.7
		20 g ha^{-1}	1051.2	66.5	83.6	11.0	5.4
		40 g ha^{-1}	2924.4	57.3	90.9	5.3	3.8
Hard wheat	Sodium selenite	0 g ha^{-1}	66.6	nd	nd	nd	nd
		10 g ha^{-1}	153.6	43.6	79.5	20.5	0
		20 g ha^{-1}	254.8	50.0	91.6	8.4	0
		40 g ha^{-1}	430.4	62.7	97.8	2.2	0
	Sodium selenate	10 g ha^{-1}	266.8	66.2	90.6	7.0	2.4
		20 g ha^{-1}	820.0	62.2	95.9	2.2	1.9
		40 g ha^{-1}	1383.2	56.4	96.5	1.9	1.6

CONCLUSIONS

Chickpeas and hard wheat could be very good candidates to be included in Se biofortification programs. But although chickpea accumulated significantly more total Se in the grain than hard wheat, the proportion of Se-Met, an effective form to increase human plasma/serum Se, was much higher in hard wheat. In terms of recommendations for a possible Se biofortification program under Mediterranean conditions, it is important to indicate that sodium selenate was much more effective, and a dose of 10 g Se ha^{-1} of hard wheat and chickpea would provide in an average diet about 80 µg Se day^{-1} and 27 µg Se day^{-1} respectively, mostly as Se-Met.

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Calcium Biofortification of Apples – Implications on Fruit Quality Parameters

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INTRODUCTION

Calcium is an essential nutrient for humans, having key structural and signalling roles (Dodd et al., 2010). Likewise, biofortification of apples with Ca can be a good method to enhance human intake of Ca (Dayod et al., 2010). Considering Ca biofortification in Golden and Jonagold apple varieties, this study aims to assess the deposition of this nutrient inside the fruits, as well as the effect of Ca-sprays treatments on the related quality parameters at harvest.

METHODS

The apple varieties Golden Delicious (G) and Jonagold (JG) were sprayed 10 times before harvest, being applied the following treatments: TC - (control treatment - 0.5% Ca $(NO_3)_2$ + 0.4% $CaCl_2$); TA (0.35% $CaCl_2$ + 1.6% $CaCl_2$) and TB (0.5% $Ca(NO_3)_2$ + 1.6 % $CaCl_2$). All samples were analyzed with the respective epidermis. At different stages of fruit growth, Ca was measured by atomic absorption spectrophotometry. Scanning electron microscopy studies were performed between the epidermis and the heart of the fruits, using a Jeol 330 coupled with a X-ray microanalyser Tracor Northern Series II. All analysis were performed at 25 Kv, 35x magnification, using a static beam spot under standardized conditions. The following physicochemical characteristics of the apples were measured: flesh firmness (using a Penefel penetrometer and expressed in Newton), color (with a Minolta CR300 Colorimeter and expressed in ºHue), total soluble solids (using a manual refractometer and expressed in ºBrix) and titratable acidity . From the data on sugar and acid content Thiault's quality index was determined (QI = total sugar g/L + 10 x total acid g malic acid/L). Statistical analysis was performed by one-way Anova ($P \leq 0.05$). For mean comparison, a Tukey test was applied, using a 95 % confidence level. Different letters indicate significant differences among treatments.

RESULTS AND DISCUSSION

At harvest, on a fresh weight basis, Ca content in Golden apples (Table 1) was significantly higher in TB (relatively to TA and TC), but in Jonagold larger amounts of Ca occurred in TA. Moreover, Ca deposition in both apple varieties remained largely distributed between the epidermis and the spongy pulp until the heart of the fruits (Fig. 1A,B). At harvest, the flesh firmness of Jonagold apples was higher in fruits of TA, followed by TB and TC (69, 67 and 62 N, respectively). A similar trend, but less pronounced, was observed for Golden variety (64, 63 and 62 N, respectively). In terms of color, no significant differences among treatments were observed for Golden apples (about 111 ºHue); however, the red color was intensified in Jonagold apples subjected to treatments TA and TB, compared with the control TC (54, 64 and 70 ºHue, respectively). The quality attributes of apples, for both varieties and different treatments, are presented in Table 2.

Table 1. Calcium contents in Golden (G) and Jonagold (JG) apples submitted to treatments TA, TB and TC, at harvest.

	Treatments (mg /100g_{fw})					
	G-TA	G-TB	G-TC	JG-TA	JG-TB	JG-TC
Harvest	3.3 ± 0.1^{b}	4.5± 0.1^{a}	3.1± 0.0^{c}	4.3 ±0.1^{a}	3.3 ± 0.0^{b}	3.0 ± 0.1^{c}

Each value is the mean ± S.D (n=2) of 15 apples. Different letters (a,b,c) indicate the significant differences ($p \leq 0.05$) among treatments within each variety.

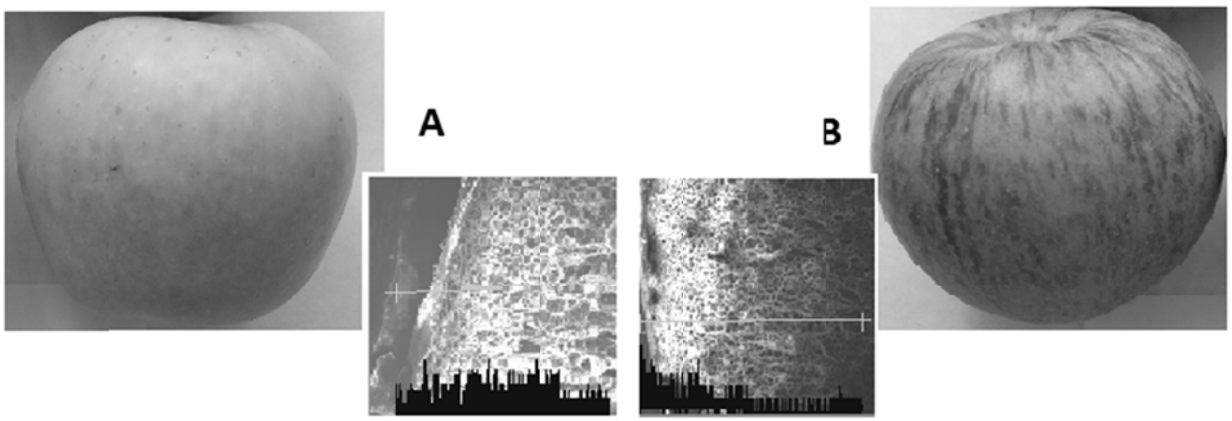

Fig.1. Scanning electron microscopy between the epidermis and the heart of Golden (A) and Jonagold (B) apples, using a Jeol 330 coupled with X-ray microanalysis.

Table 2. Quality attributes of fruits submitted to treatments TA, TB and TC at harvest.

Variety	Treatment	Quality indicators			QI**
		Acidity (g malic acid/L)	RI (ºBrix)	TSS – Total Soluble Solid* (g/L)	
Golden	TA	4.9	12.4	110.5	159.7
	TB	5.3	13.8	125.3	178.2
	TC	4.4	13.3	120.0	164.5
Jonagold	TA	5.8	16.3	152.1	211.1
	TB	4.7	15.6	144.5	192.0
	TC	5.0	13.3	119.9	169.8

* Golden delicious (NF V20-201) norm was used to convert RI to TSS (http://www.sensnco.fr/m-137-les-normes.html).
** QI (quality index or Thiault index) defined for Golden delicious (J. Thiault, 1970).

CONCLUSIONS

Considering Ca biofortification, the overall observations suggest that TB was better for Golden apples and TA for Jonagold apples, but Ca largely spreads within the pulp of both varieties. In general, when compared to the control (TC), Ca-sprays enhanced physicochemical and quality attributes of both apple varieties.

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Zinc Requirements from Biofortified Crops: How Important is Bioavailability?

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The efficacy of zinc biofortification of crops providing major food staples to poor populations depends not only on the increase in zinc concentration of the grain but on the bioavailability of that zinc. It also depends on zinc requirements (quite apart from bioavailability) which have been the focus of considerable debate recently and the quantity of grain consumed. This presentation will be based on four of our studies of zinc bioavailability. These have included three studies of zinc biofortification, including one that also involved iron biofortification and another that included zinc fortification in addition to biofortification. The fourth study was directed to maize with low phytate levels achieved with selective breeding.

In each of these studies, the three meals consumed during a single day, the zinc intake from which was provided by the zinc biofortified or control grain, were extrinsically labelled with a zinc stable isotope and fractional absorption measured using our dual isotope ratio technique. Dietary zinc intake was measured from duplicate meals and the total quantity of zinc absorbed (TAZ) was determined from the product of these 2 measurements.

In one study, TAZ was determined from zinc biofortified wheat administered to healthy adult women. The additional quantity of zinc absorbed compared with that for controls was that predicted utilizing our trivariate model of TAZ as a function of dietary zinc and dietary phytate. TAZ depended, as predicted, on the extent to which the grain was milled. This study demonstrated that the bioavailability of the additional zinc obtained by biofortification was indistinguishable from that in the control grain and also demonstrated the feasibility of predicting the gain in zinc absorption from biofortification in healthy subjects whose intake of the grain had been / could be approximately estimated.

Another study was directed to children aged 2 yrs in rural N. Karnataka, India who were fed a zinc and iron biofortified pearl millet, with a zinc intake averaging 5.8 mg Zn/day for the biofortified group 3.3mg Zn/day for controls. In contrast to those children randomized to the control grain group, the TAZ of the zinc biofortified group exceeded the estimated physiological requirement of the Institute of Medicine, USA. However, our research use of Saturation Response Modelling (SRM) for broadening our understanding of zinc homeostasis and requirements yielded what appears to be a very abnormal pattern. This pattern was consistent with impaired bioavailability, presumably at least in part due to the high phytate intake, but possibly also to host gut factors. The model was also consistent with the concept of 'pathophysiological' requirements perhaps due to excessive endogenous zinc losses and also to depleted zinc stores. This, however, remains conjecture at this time.

A third zinc biofortification bioavailability study has been undertaken in children aged 2 yrs in rural Zambia. Milling of the maize with an open hammer mill, as is a common practice in this population, removed all zinc, but the zinc was retained well when the grain was roller milled. Results of this study, which also included a zinc fortification arm, will be presented.

Also pertinent to this research, are the results of longitudinal determination of TAZ across pregnancy and lactation in indigenous Guatemalan women. TAZ increased reaching a peak in early lactation and closely matched the predicted increases in physiological requirements during the reproductive cycle. These results are consistent with finely tuned up-regulation of zinc absorption to match the increased requirements, an up-regulation that was not affected by phytate content of the maize.

An additional pertinent recent event has been the correction of erroneously very low estimates of zinc physiological requirements that had been utilized by HarvestPlus in setting goals. Within the past 2 years, these have been corrected by a workshop convened by HarvestPlus and, more recently, by

European DRVs. Coupled with our concept of 'pathophysiological' requirements in at least some populations in most need of zinc biofortification of food staples, a reasonable conclusion is that goals for biofortification should not be set on the basis of estimates of human physiological requirements. Rather goals could best be set by agronomic limitations.

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The HarvestPlus Update on Nutrition Studies in Biofortified Crops

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INTRODUCTION

The goal of HarvestPlus is to improve the health of poor people by breeding staple food crops that are rich in micronutrients, a process referred to as *biofortification*. Micronutrient malnutrition, primarily the result of diets poor in bioavailable vitamins and minerals, affects more than half of the world's population, especially women and pre-school children. The biofortification strategy seeks to take advantage of the consistent daily consumption of large amounts of food staples, thereby providing a nutritional boost that was previously not available. Measuring the nutritional benefits of biofortified crops has been a major work-in-progress since the advent of the HarvestPlus program. Outcomes of these investigations, including new lines of research will be presented.

Efficacy studies on biofortified crops (rice, bean and pearl millet) have been undertaken in the Philippines (rice), Mexico (beans) and India (Pearl Millet). Individuals within these studies were found to vary in the incidence of Fe deficiency, with 31% in the rice study being Fe deficient, 11 and 71% were found to be Fe deficient in bean studies conducted in Mexico and Rwanda respectively, while in a pearl millet study, 44 % of subjects were considered Fe deficient. The increased intake of Fe from biofortified foods lead to increased absorbable Fe which in turn improved various biochemical markers such as ferritin and/or total body Fe. The extra Fe from biofortified crops was predicted to meet a good portion of the estimated average requirements.

Zn-efficacy studies are underway or in the planning stages. Two Zn efficacy studies in wheat will include a major study in the slums of New Delhi and a study with women of reproductive age. For rice, a feeding trial in women and children is in the planning stages and expected to be completed in 2015.

In terms of breeding for improved Fe or Zn bioavailability, there are preliminary investigations underway to analyze association mapping panels of wheat and rice for total absorbable Zn (TAZ) (Miller et al., 2007) and if these mapping studies determine that there are significant associations between TAZ and SNPs, and the trait is of a high heritability, then this will be explored further for validation purposes and one might see further work in developing breeding material with a higher TAZ.

In conclusion, there is emerging evidence of the benefits of improved Fe nutrition through Fe-biofortified crops. Major Zn-efficacy studies currently or soon to be underway, will hopefully tell a similar story. Breeding efforts are advanced for improving the seed nutrition of many crops but at this stage, little research in improving bioavailability has been undertaken. Pilot programs to map TAZ in wheat could lead to new programs in targeting bioavailability as a breeding trait.

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Effect of Different Cereals and Soil Contamination in Malawi Diets on Potential Iron Availability: Results from an in vitro Caco-2 Cell Model

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INTRODUCTION

Iron deficiency is usually associated with the consumption of plant-based diets as these provide lower quantities of bioavailable iron than diets containing meat, poultry and fish. A recent cross-sectional study was carried out in two regions of Malawi: Mikalango, where the main cereals are sorghum and pearl millet, and Zombwe, where unrefined maize is the main cereal. Iron intakes (estimated from analysis of composite diets) were significantly higher in Mikalango than Zombwe (29.6 vs. 16.6 mg/d) but body iron levels were significantly lower in women aged 18-50y (Siyame et al 2014). We tested the hypothesis that the discrepancy between iron intake and iron status between regions was due to differences in the iron bioavailability of the two diets and the contribution of additional iron from soil, based on high levels of Al and Ti in the diet composites. It has been suggested that contaminant iron from soil makes a useful contribution to total iron intake in Ethiopia (Gebre-Medhim et al 1976).

METHODS

An in vitro Caco-2 cell model was used to assess iron availability from composite diets and soil samples collected in Mikalango and Zombwe (Siyame et al 2014). Weighed duplicate diet composites (including drinking water) were collected for one day. The iron content of the diets was measured by ICP-MS and the soils by ICP-MS and XRF. Analysis of phytate was performed by HPLC. The soil samples from Zombwe (acidic) and Mikalango (calcareous), were composited as regional samples and autoclaved. Food and soil samples were subjected to a simulated gastrointestinal digestion (Glahn et al 1996) and the digestate applied to a monolayer of Caco-2 cells (passage 28-30) 13d post seeding (Wawer et al 2012) together with ascorbic acid (AA) (1:30 iron:AA molar ratio) to improve the sensitivity of the model by increasing uptake of iron into the cells. The Caco-2 cells were exposed for 2h to the digestate and 22 h later the cells were harvested, sonicated and analysed for ferritin (Ramco, USA) and protein (Pierce, USA). Uptake of iron by Caco-2 cells, a surrogate index of iron availability, was estimated from the ferritin content (ng/mg total protein) (Glahn et at 1998).

RESULTS AND DISCUSSION

The iron content of composite diets and soils and estimated daily intakes are shown below:

	Mikalango	**Zombwe**
Iron content of composite diet sample (μg/g)	94.8	58.6
Iron content of composite soil sample (mg/g)	47.1	25.8
Estimated iron intake (mg/d)	29.6	16.6
Estimated phytate intake (mg/d)	1564	846

There was no ferritin response in cells treated with composite diets at any concentrations, probably due to the solubilised iron binding to phytate, high in diets from both regions, and to polyphenols, present in the sorghum (red) and millet varieties consumed in Mikalango.

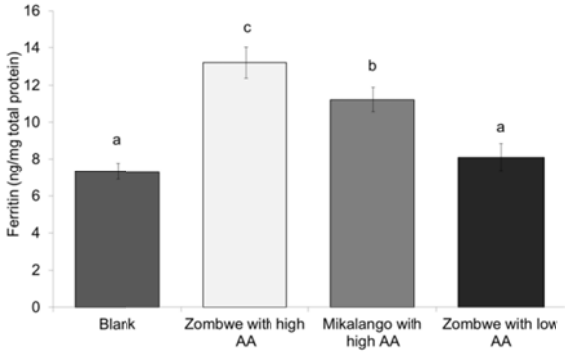

Fig 1. When Caco-2 cells were treated with similar weights of soil samples, significantly more ferritin was produced with digests of Zombwe than Mikalango soil ($p<0.005$)

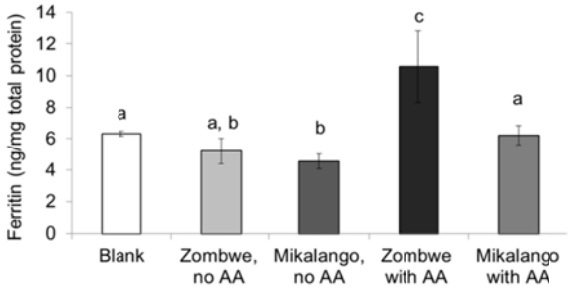

Fig 2. When the weight of the soils was adjusted in order to expose the cells to similar quantities of iron from digestates of the two soils (8.29 mg iron/well), there was a greater ferritin response with the soil from Zombwe than Mikalango with high AA ($p=0.01$)

CONCLUSIONS

The high phytate content of the diets in both regions of Malawi would suggest low iron availability and probably explains the lack of ferritin response in the Caco-2 cell model. The acidic soils of the Zombwe region contained more available iron than the calcareous soils of Mikalango (Fig 1), and even when similar concentrations of iron were applied to Caco-2 cells, the digestate from Zombwe elicited a greater ferritin response (Fig 2), suggesting there may be other modulators of iron availability in the soils, for example differences in iron mineralogy or calcium concentrations. The presence of acidic contaminant soil in processed foods may contribute to dietary iron intake and make a useful contribution to iron nutrition.

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Nutritional Quality of Selected Lettuce and Endive Cultivars Enriched With Mg

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INTRODUCTION

The challenges of modern plant production is to adapt itself to the increase of world population and to meet consumers new requirements and food preferences, which are food products with high nutritive value, natural and free from chemical additives. Therefore, production of leafy vegetables or sprouts enriched with essential elements is deservedly receiving an increasing attention (Ramos *et al*. 2010, Smoleń *et al*. 2014). As magnesium (Mg) deficiency is common in many developed countries (Johnson 2001) and lettuce is one of the most willingly consumed leafy plant the aim of this work was to assess the possibility of enriching Romaine lettuce (*Lactuca sativa* L. var. *longifolia* Lam.), head lettuce (*Lactuca sativa* L. var. *capitata*) and endive (*Cichorium endivia* L.) plants with Mg ions.

METHODS

The study objects were plants of Romaine lettuce (*cv*. 'Amadeusz'), head lettuce (*cv.* 'Omega') and endive (*cv.* 'Burundi'). Plants were grown in spring in hydroponic culture (composition of nutrient solution provided by growers) for 6 weeks at pH 6.0-6.2, photoperiod 10/14 h day/night and temperature 20/14°C. Mg was applied in the last 4 weeks of cultivation in form of $MgSO_4*7H_2O$ at concentrations of 40 (control), 80, 120 and 160 mg dm^{-3}. During plants' growth (i) gas exchange, (ii) chlorophyll *a* fluorescence, (iii) total chlorophyll content and (iv) transpiration rate were examined. At harvest, data on (v) biomass accumulation, (vi) levels of ROS, superoxide anion-radical ($O_2°^-$) and hydroxyl radical (OH°), (vii) activity of enzymes of anti-oxidative system, ascorbate peroxidase (APX) and catalase (CAT) and (viii) selected ions content were recorded.

RESULTS

Addition of Mg to growing medium increased biomass accumulation in the aboveground parts, both fresh weight and dry matter, in 'Amadeusz' and 'Burundi', and decreased (fresh weight) or did not change (dry matter) in 'Omega' (Table 1).

Table 1. Fresh weight and dry matter of leaves of lettuce and endive plants grown in nutrient solution enriched with Mg ions. Data are mean ±SE, n=5.

Cultivar	Measured parameter	Mg concentration (mg dm^{-3})			
		Control (40)	80	120	160
'Amadeusz'	Fresh weight (g)	208.9	223.3	225.5	238.1
	Dry matter (g)	20.9	21.7	22.5	22.3
'Burundi'	Fresh weight (g)	120.8	158.4	146.7	159.7
	Dry matter (g)	8.4	9.9	8.3	9.3
'Omega'	Fresh weight (g)	226.2	230.3	207.4	180.3
	Dry matter (g)	10.4	11.0	10.1	10.9

Increased biomass production due to Mg ions in 'Burundi' plants was, at least partially, attributed to greater intensity of photosynthesis. In 'Omega' plants intensity of this process was decreased and in 'Amadeusz' unchanged. These results corresponds well with data on intensity of transpiration and stomatal conductance, which were higher in 'Burundi', almost unchanged in 'Amadeusz' and lowered in 'Omega'. Effect of Mg on parameters of chlorophyll *a* fluorescence was rather slight. However, it is worth mentioning that in 'Burundi' plants Mg ions increased overall quantum yield of photochemical energy conversion in PS II (Yield) and photochemical quenching (qP), and decreased nonphotochemical quenching (qN, NPQ). In 'Amadeusz' and 'Burundi' chlorophyll content was higher in plants grown in the presence of Mg, while in 'Omega' this parameter was not changed.

Level of examined ROS in 'Burundi' plants increased due to Mg ions, in 'Amadeusz' level of $O_2^{\bullet -}$ increased and OH° decreased, while in 'Omega' level of ROS was always reduced. In 'Omega' most probably other, not measured, components of oxidative stress were induced. Activity of enzymes of antioxidative system increased in 'Burundi'. In 'Amadeusz' Mg treated plants higher activity of CAT and lower of APX was recorded. In case of 'Omega' obtained results were less evident, but slightly greater activity of APX and lower of CAT were noted.

Addition of elevated doses of Mg increased concentration of Mg, while decreased of Fe and Mn (Table 2). Concentration of Zn was greater in Mg treated 'Burundi' plants, and usually decreased in 'Amadeusz' and 'Omega'. 'Burundi' grown in the presence of Mg had higher concentration of Ca, in 'Amadeusz' it was unchanged and lowered in 'Omega'. Worth emphasizing is the fact that 'Omega' plants had reduce uptake of all examined elements, except Mg (Table 2).

Table 2. Selected ions content in leaves of lettuce and endive plants grown in nutrient solution enriched with Mg ions. Data are mean ±SE, n=3.

Cultivar	Mg concentration (mg dm^{-3})	Examined element (mg g^{-1} DW)				
		Mg	Fe	Mn	Zn	Ca
'Amadeusz'	Control (40)	1.47	0.052	0.109	0.020	8.32
	80	2.18	0.041	0.091	0.017	8.85
	120	2.58	0.048	0.097	0.018	8.26
	160	2.61	0.044	0.085	0.017	8.12
'Burundi'	Control (40)	3.00	0.103	0.128	0.019	11.08
	80	3.63	0.070	0.111	0.020	10.89
	120	4.29	0.073	0.112	0.020	11.93
	160	4.64	0.105	0.118	0.022	12.81
'Omega'	Control (40)	2.55	0.074	0.125	0.022	10.13
	80	3.25	0.065	0.120	0.020	9.32
	120	3.67	0.054	0.133	0.025	9.49
	160	2.91	0.046	0.122	0.020	6.98

CONCLUSIONS

Of the species and cultivars tested, 'Burundi' proved to be the most sufficient for enrichment with Mg ions. 'Burundi' plants grown in the presence of Mg in concentration up to 120 mg dm^{-3} accumulate considerable amounts of Mg, without causing negative effects on examined physiological processes and aggravation of oxidative stress.

ACKNOWLEDGEMENTS

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Session-IV:
STSM Presentations

Effect of Nitrogen Soil Application on Zinc and Iron Concentration in Maize Plants

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INTRODUCTION

Micronutrient malnutrition is a growing concern in developing countries, where this problem is present in human but also in animal nutrition. Increasing the Zn and Fe concentration of food crop plants, resulting in better crop production and improved human and animal health is an important global challenge. Among micronutrients, Zn deficiency occurs in: crops, animals and humans (Cakmak, 2007). Zinc deficiency is currently listed as a major risk factor for human health and cause of death globally. According to a WHO report (2002) on the risk factors responsible for development of illnesses and diseases, Zn deficiency ranks 11th among the 20 most important factors in the world and 5th among the 10 most important factors in developing countries. Increasing Zn concentration of grain (or other edible parts) by soil and/or foliar applications of Zn also brings several agronomic benefits for crop production. Applying Zn to plants grown under potentially Zn-deficient soils is effective in reducing uptake and accumulation of P (and thus phytate) in plants. This agronomic side effect of Zn fertilization may result in better bioavailability of Zn (Cakmak, 2010).

In modern ruminant nutrition, micronutrients are added mainly through different types of additives in concentrate part of ration. Other part of ration which is most commonly maize silage can also be used for micronutrient supply if we use micronutrient enriched maize for silage production. By using different ways of fertilization, for example, by applications of N fertilizers trough soil and foliar application we can achieve positive effect on Zn content in plant (Kutman et al., 2010) and probably other essential micronutrients. Achieving the increase of Zn and Fe content in maize plats through agronomic biofortification could result in higher content of Zn and Fe in ruminant ration and as a result reduction or exclusion of inorganic and chemical additives and by that have positive cost effect.

METHODS

Experiment with maize was set as pot experiment in green house controlled environment. Main aim was to see effect of N fertilization on Zn and Fe uptake and concentration in the maize plants and in addition Se treatments were included. Soil used for experiment was deficient with trace elements especially Zn and Fe. Total number of pots was 56 with 3 kg of soil in each pot. Application of N, Zn and Fe fertilizers was made and they were mixed with soil before seeding. N was added in the form of $Ca(NO_3)_2$ in high (250 ppm N/kg soil) and low (125 ppm N/kg soil) doses. Zn was added in the form of $ZnSO_4$ in bought N treatments in high (5 ppm Zn/kg soil) and low (1 Zn ppm/kg soil) doses. Fe was added in the form of Fe sequestrated (10 ppm Fe/kg soil) to the treatments. Se was added in the form of Na_2SeO_4 (0,02 ppm Se/kg soil). Deionized water was used for watering. Plants were grown for 25 days after which they were harvested, dried and digested. Measurement of the concentration of Zn, Fe and other nutrients was done according to ICP-OES methodology.

RESULTS AND DISCUSSION

Results show that initial hypothesis of positive effect of N fertilization on micronutrient concentration in maze plants is justified.

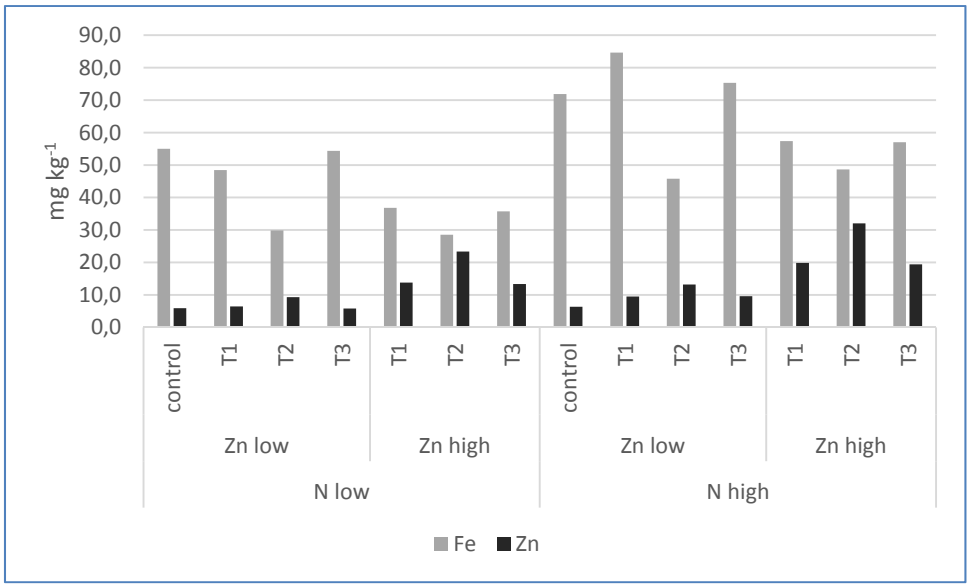

Fig. 1: Concentration of Zn and Fe in maize plants undre diferent N and Zn conditions.

CONCLUSIONSC

Due to results obtained in greenhose experiment and similar research done by other scientists it can be expected that agronomic fortification of Zn and Fe in maize with N can be wery effective. This can be very valauable information in proces of silage maise production for dairy cow nutrition.

ACKNOWLEDGEMENTS

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Influence of Foliar Sprays on Photosynthesis in Grapewine

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INTRODUCTION

In the time of high level of informational awareness and easy access to the information, the selection of high quality information is difficult. As a consequence, it often happens that farmers don't follow the guidelines of experts about pest control agents or use of foliar fertilizers.

Because of foliar application sun irradiation is reduced and consequently photosynthesis especially in years, when during the period of grape ripening we encounter increased levels of rainfall and decreased time of solar irradiation. This might influence the quality / quantity of product, but unfortunately the problem is not well studied.

Response to foliar nutrient sprays can be remarkable when treatments are applied to deficient plants (Neumann, 1988). Indeed, the majority of agronomic foliar nutrient spray studies have been developed with deficient plants and foliar fertilizers have been traditionally used to correct punctual nutrient deficiencies. However, there is an increasing trend to apply foliar sprays in the absence of deficiency symptoms, at least as it refers to elements with little phloem mobility (Fernández, 2009). Therefore the need to study unnecessary use of foliar sprays emerges.

There is also evidence that interactions between foliar spray additives occur, but nowadays it is not yet possible to predict the performance of a certain active ingredient in combination with a particular surfactant (Fernandez, 2005).

METHODS

The experiment was performed in the 10 years old vineyard with the grape variety '*Chardonnay*' in leaves (3 plants, 3 replicates) in the first day without any application at two time intervals: first measurement from 10 to 12 am, when the stomata are opened and second measurement from 14 to 16 am, when the stomata are closed.

Then surface of the leave has been intensively sprayed at the end of the first day and let dried. Leaf gas exchange has been measured prior and after application of water, pest control agent CuSO4 (Calda Bordalesa), at 20% of Cu - 20 g/L, mineral fertilizer (Basfoliar, 3 ml /l), organic compounds (humic acid, 100 mg /l).

Determinations of stomata conductance, net photosynthesis and internal CO_2 were performed using a CIRAS-1 (PP Systems, UK) system, under environmental irradiance and temperature; CO2 set to around 380-390 ppm. All values have been calculated relative to control plant (even before application, to eliminate environmental factors).

RESULTS AND DISCUSSION

Stomatal conductance show similar response regardless the material applied (except water, which shows no effect anywhere): decreased values. The change is bigger in the afternoon, when the stomata are closed. The decrease of the net photosynthesis after application is smaller, but still significant, when the stomata are closed, but not present, when they were open regardless which application we have used.

Differences between applications have been observed only for the internal CO_2 production, where mineral fertilizer caused decrease, but organic matter increase in internal CO_2 production, while and

pest control agent decreased internal CO_2 production when the stomata were closed and increase, when the stomata were open.

Strong decrease in CO_2 uptake and stomatal conductance has as well been observed at the grapevine variety 'Chardonnay', exposed to relatively weak UV irradiation. This is similar as in our case with mineral fertilizer application and opposite as for organic matter application rising additional questions about the similarities, but also a possibility to use organic matter to “neutralize” eventual UV-B effects.

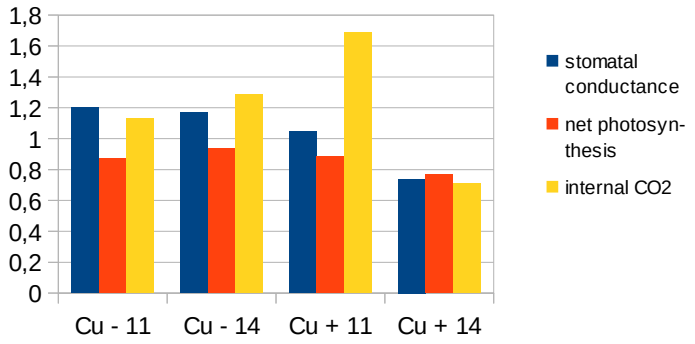

Fig. 1: Relative values of stomatal conductance, net photosynthesis and internal CO_2 (compared to control) prior to and after the treatment with $CuSO_4$, with the stomata open (11) and closed (14).

CONCLUSIONS

Research, conducted during STSM indicate some interesting possibilities, worth further investigation. Different behavior for each application indicate, that foliar application should be more intensively studied. Applications have bigger influence on the photosynthesis, when stomata are closed. This can be actually useful, especially in extremely hot conditions and is already under investigation.

But for our farmers, results with the stomata open are more interesting, since our main concern is extreme application in more rainy conditions. Therefore a multi factor experiment has been set in 2013, to study the changes in more details, but the results are still under evaluation.

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Survey of Barley Genotypic Variation in Re-translocation of Foliar Applied Zn, Fe and Se

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INTRODUCTION

To lead a healthy life humans need adequate nutrient supply (Bouis and Welch, 2010). However, many populations suffer from malnutrition due to shortage in essential elements. Agricultural measures such as crop selection, fertilizers, cropping systems, and soil amendments could be used to increase nutrient outputs of farming systems and guarantee a balanced micronutrient status (Graham et al., 2007). Biofortification of staple food crops with micronutrients through plant breeding and/or agronomic tools is probably the most sustainable and cost-efficient strategy to avoid micronutrient shortage (White and Broadley, 2005; Pfeiffer and McClafferty, 2007; Cakmak 2008). Biofortifying means to increase the micronutrient level in crop edible parts to amounts improving the nutritional health of individuals that would otherwise run a high risk of developing malnutrition. Genotypes of a given plant species may greatly differ in their capacity to accumulate micronutrients in their edible parts such as seeds/grains (Gomez-Becerra et al., 2010a). Understanding differential genetic expression of micronutrient acquisition in seeds would contribute to better control of the biofortification process. Since barley was the fourth important crop worldwide in terms of quantity produced and its area of cultivation (FAOSTAT) in 2010, attention has been focused on accumulation of selected micronutrients (such as Zn, Fe and Se) in different barley cultivars. These three micronutrients are of great importance for human health at global level, affecting more than 3 billion people worldwide, particularly in developing countries (Cakmak et al., 2010a). Zn and Fe deficiencies are known as the most prevalent micronutrient deficiencies, causing serious health complications (Cakmak et al., 2010a), whilst Se is an essential trace element in many enzymes. Plant-delivered foodstuffs, namely cereals, are the major dietary sources of these minerals in most countries throughout the world.

METHODS

Genotypic variation in re-translocation of foliar applied micronutrients was investigated in barley by using one Bulgarian (Aster) and three Turkish (Tokak, Ince and Larende) barley cultivars. Barley genotypes were grown under adequate nutrient supply in an experimental soil under greenhouse conditions at Sabanci University. The soil was calcareous with a sandy clay loam texture, high pH (8.2) and very low organic matter content (0.62 %). Zinc concentration extracted by DTPA was 0.07 mg kg^{-1} soil. To avoid Zn deficiency stress in plants, all pots have received sufficient amount of Zn (2 ppm) in form of $ZnSO_4$. At maturation, plants were harvested and grain yield and grain concentrations of micronutrients were determined. Grain samples were acid-digested in a closed-vessel microwave system (MarsExpress; CEM Corp., Matthews, NC, USA). The concentrations of all mineral nutrients were determined by ICP-OES (Vista-Pro Axial; Varian Pty Ltd, Mulgrave, Australia). The samples digested for the mineral nutrients analysis were used also for the determination of Se concentrations by AAS (Spectra AA 220-FS, Agilent, Australia) equipped with VGA 77 (vapor generation accessories) and ETC-60 (electrotermal temperature controller). In addition, in selected samples Se species analysis was conducted by using the methods described by Solovyev et al. (2013). *Statistical Analysis:* Concentrations after treatment were compared to concentrations in the control group via two-sided t-tests. All analyses were performed with the software r, p-values below 0.05 are considered significant.

RESULTS AND DISCUSSION

Foliar applications of $ZnSO_4$ (e.g., at booting and early milky stage) caused marked increases in grain Zn concentrations in all genotypes tested. The highest Zn grain concentration was measured in the barley genotype Aster (36 ppm Zn) followed in decreasing order by Tokak (34 ppm Zn) and Ince (34 ppm Zn), and the lowest increase was found in Larende (31 ppm Zn). The control plants (no Zn spray) had 15, 17, and 18 ppm Zn in grain for the genotypes Aster, Inge + Tokak, and Larende, respectively. Comparison among all tested genotypes showed that Zn spray in combination with Na selenate leads to additional increases in grain Zn concentration. Among all genotypes tested, the barley genotype Ince showed the highest Fe concentration after foliar FeEDTA application (46 ppm Fe) while control plants had 37 ppm Fe. In the genotypes Aster and Tokak the combination of $ZnSO_4$+Na selenate+FeEDTA increased additionally the Fe concentration in comparison to single foliar FeEDTA application.

Following the Na-selenate spray to foliar, the barley genotypes Ince (3.9 ppm Se), Tokak (3.7 ppm Se), and Larende (3.5 ppm Se), accumulated much higher Se compared to the genotype Aster (2.4 ppm Se). No-Se-sprayed-plants had 0.04, 0.05, 0.06, and 0.09 ppm Se in grain for the genotypes Aster, Inge, Tokak, and Larende, respectively. Selenium speciation analysis showed existence of the following Se-species in the Se-biofortified barley grain: Se-protein-P (SePP), GPx (glutathione peroxidase), Se-cystein (SeCys) and Se (VI). The genotypes studied showed significant genotypic variation regarding composition and amount of the investigated Se-species.

CONCLUSIONS

Foliar spray of Zn and Se substantially increased their respective concenrations in grains, while Fe spray remained less effective. $ZnSO_4$ and Na-selenate can be sprayed together without any adverse effects on their grain contents. Genotypes tested were clearly different in their response to foliar spray of Zn, Fe and Se. Further investigations on the effects on food quality are foreseen.

ACKNOWLEDGEMENTS

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Transcriptome Differences Between Accessions of *N. caerulescens*

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INTRODUCTION

Noccaea caerulescens is a hyperaccumulating plant that has a high degree of intra-specific variation in its metal accumulation and tolerance properties. Different accessions of *N. caerulescens* are able to accumulate and tolerate different levels of zinc, nickel and cadmium (Assunção *et al*., 2003). High throughput sequencing of the root and shoot transcriptomes of four accessions of *N. caerulescens,* which have well characterised phenotypes, can provide information about the differences that contribute to the metal accumulation and tolerance traits. RNA sequencing enables the analysis of gene expression levels as well as sequence and splicing differences.

METHODS

The root transcriptomes of three accessions of *N. caerulescens* Ganges (GA), Monte Prinzera (MP) and La Calamine (LC) were sequenced using SOLiD sequencing and aligned to the *Arabidopsis thaliana* genome using Bioscope 1.3.1. Read counts were obtained for each gene and then tested for differential expression in pairwise comparisons using Limma (Smyth, 2005). The expression pattern of the different accessions was used to link the genes to an accumulation or tolerance trait based on the phenotype of the accessions. In addition, root and shoot transcriptomes from four accessions of *N. caerulescens* GA, MP, LC and Lellingen (LE) were sequenced using Illumina sequencing. The RNA sequence data for each accession was assembled using the Trinity *de novo* assembler (Grabherr et al., 2011) producing a transcriptome for each accession. Genes that are of interest in metal accumulation and tolerance can be identified and compared for sequence differences between the accessions.

RESULTS AND DISCUSSION

1,200 genes were identified from the SOLiD sequencing that were differentially expressed in one or more of the pairwise comparisons. GO analysis of these genes revealed that the most significant terms were related to metal ion transmembrane transporter activity. Analysis of the IRT1 gene in *A. thaliana*, GA, LE and MP accessions using the assembled RNA sequence data revealed differences in the amino acid sequence of the protein (Fig. 1).

With the availability of the *N. caerulescens* genome sequence it should be possible to increase the power of the analysis. When mapping to the *A. thaliana* genome, areas of the reference that have a large number of differences to the studied species are not mappable. Mapping to the *N. caerulescens* genome will increase the amount of usable data and could reveal new information. A genome-guided assembly of the RNA data may also produce a more accurate transcriptome. An ongoing analysis is looking at the expression level and sequence differences in the shoot transcriptome data of the four *N. caerulescens* accessions.

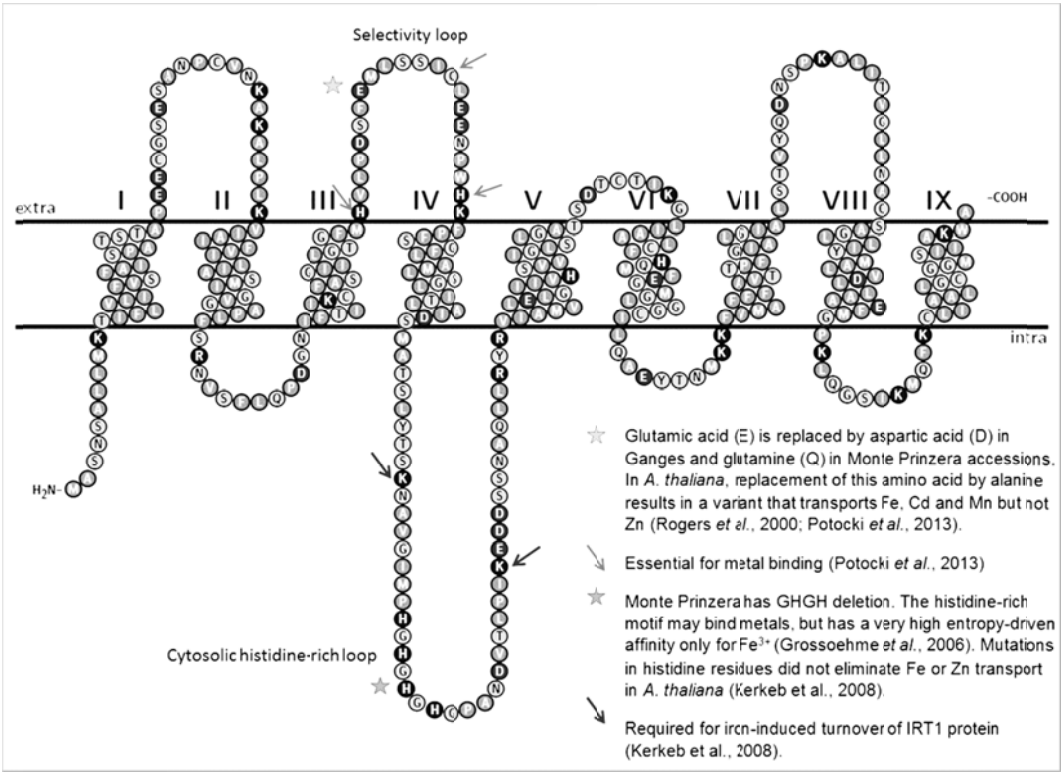

Fig. 1: Predicted secondary structure of the *A. thaliana* IRT1 metal transporter using HMMTOP (Tusnády and Simon, 1998) and textopo (Beitz, 2000). Amino acids known to be important for function and also areas where the *N. caerulescens* accessions differ are labeled. (Halimaa *et al.,* submitted).

CONCLUSIONS

RNA sequencing can reveal information about gene expression levels and the nucleotide sequence of a sample. By mapping reads to a close reference species, genes that are differently expressed and have sequence differences between accessions can be identified. When a reference sequence becomes available later, additional information can be obtained from the data.

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Expressing *NcZNT1* and *NcMTP1* from *Noccaea* (*Thlaspi*) *caerulescens* Enhances Zn and Cd Tolerance and Accumulation in *Nicotiana tabacum*

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INTRODUCTION

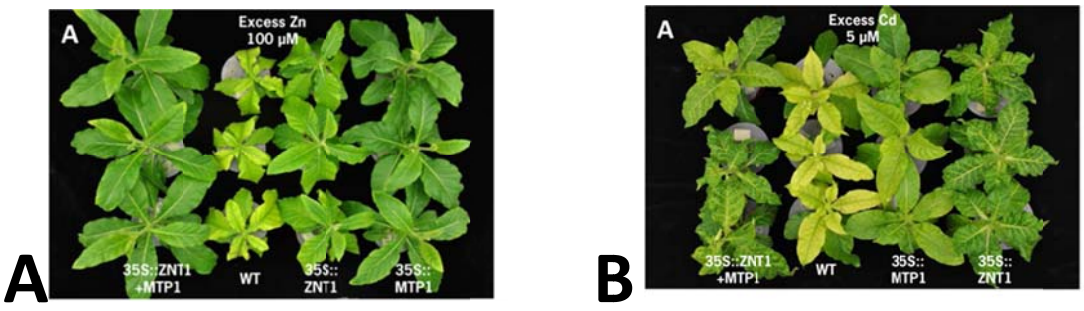
Zinc (Zn) and cadmium (Cd) metals contaminate soils when present in excess. Phytoremediation could potentially be an efficient tool to remediate the metal contaminated soils (McGrath and Zhao, 2003). *Noccaea (Thlaspi) caerulescens* is a model metal hyperaccumulator species that can accumulate up to 3% of zinc, but also high amounts of nickel and cadmium (Lombi et al., 2000). Less biomass of this species makes it unsuitable for phytoremediation purposes. This limitation can be overcome if higher biomass producing species, like *Nicotiana tabacum*, are engineered to express genes from metal hyperaccumulation species *N. caerulescens*. *NcZNT1* is a member of the *ZIP* gene family in *N. caerulescens* and is localized to plasma membrane, while *NcMTP1* is a member of the CDF gene family, predicted to localize to the vacuolar membrane (Hassan and Aarts, 2011). The current study was aimed at analyzing the involvement of *NcZNT1* and *NcMTP1* in Zn/Cd tolerance and accumulation in tobacco and to enhance the phytoremediation potential of tobacco by expressing these genes.

METHODS

Both *NcZNT1* and *NcMTP1* genes were expressed separately and in combination into *Nicotiana tabacum* under the control of constitutive CaMV 35S promoter to investigate their role in Zn, Cd accumulation and tolerance and to enhance the phytoremediation potential of this species. Single and double transgenic lines with high expression of the transgenes were grown hydroponically under excess Zn and Cd supply and were analyzed for biomass, photosynthesis capacity, lipid peroxidation, redox enzyme activities and metal accumulation. The same lines were also grown in Zn/Cd contaminated field soil to test their enhanced phytoremediation potential.

RESULTS AND DISCUSSION

Both *NcZNT1* and *NcMTP1* expressing transgenic lines were more tolerant to excess Zn and Cd and showed up to 4 fold higher Zn, and up to 3.5 fold higher Cd uptake capacities based on collective metal concentration and biomass data (Fig. 1). All transgenic lines showed better photosynthetic capacity and less oxidative damage revealed by cellular redox state data compared to the wild type. Transgenic lines exhibited metal tolerance in Zn/Cd contaminated field soil and *pro35S::NcMTP1*line showed the ability to reduce Cd pollution level of metal contaminated soil from 2.11 mg kg-1 to 1.05 mg kg-1 in about 10 generations due to its higher BioAbsorption Coefficient (Table 1). We conclude that the over expression of *NcZNT1* and *NcMTP1* improves excess Zn/Cd tolerance and enhanced Zn/Cd accumulation probably through enhanced cellular metal uptake across the plasma membrane and compartmentalization in the vacuole respectively and *pro35S::NcMTP1*line has a good Cd phytoremediation potential.



A **B**

Fig. 1: Phenotype of *pro35S::NcZNT1*, *pro35S::NcMTP1*, *pro35S::NcZNT1* + *pro35S::NcMTP1* lines compared to WT grown on excess Zn 100µM (A) and Cd 5µM (B) after growing for 4.5 weeks in hydroponics.

Table 1: BioAbsorption Coefficient (BAC), the BioConcentration Factor (BCF) and the Translocation Factor (TF) of *pro35S::NcZNT1*, *pro35S::NcMTP1* and *pro35S::NcZNT1* + *pro35S::NcMTP1* lines compared to WT grown in metal contaminated field soil.

	BAC Shoot Conc./Soil Conc. (mg kg^{-1})		BCF Root Conc./Soil Conc. (mg kg^{-1})		TF Shoot Conc./Root Conc. (mg kg^{-1})	
	Zn	Cd	Zn	Cd	Zn	Cd
WT	2.93	16.27	24.67	56.38	0.12	0.29
ZNT1	2.36	16.98	25.94	73.46	0.09	0.23
MTP1	2.68	21.95	23.47	57.56	0.11	0.38
ZNT1+MTP1	2.55	20.43	22.64	47.15	0.11	0.43

CONCLUSIONS

We conclude that the over expression of *NcZNT1* and *NcMTP1* improves excess Zn and Cd tolerance and accumulation probably through enhanced cellular metal uptake across the plasma membrane and compartmentalization in the vacuole respectively and *pro35S::NcMTP1*line has a good Cd phytoremediation potential. This line exhibited the ability to reduce Cd pollution level of metal contaminated field soil from 2.11 mg kg-1 to 1.05 mg kg-1 in about 10 generations.

ACKNOWLEDGEMENTS

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Sensing Zinc Deficiency: Analysis of a Putative Zinc-Sensor Function in AtbZIP19/23 Transcription Factors

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INTRODUCTION

Plants are capable of inducing a range of physico-chemical and microbial modifications of the rhizosphere which can mobilize mineral nutrients or prevent toxic elements from entering the roots. Understanding how plants sense and adapt to variations in nutrient availability is essential in order to develop plant-based solutions addressing nutrient-use-efficiency and adaptation to nutrient-limited or -toxic soils.

Recently two transcription factors (TFs) from the bZIP family, bZIP19 and bZIP23, have been identified in Arabidopsis and shown to be pivotal in the adaptation response to zinc deficiency [1]. They represent not only the first regulators of zinc homeostasis identified in plants, but also a very promising starting-point that can lead to new insights regarding the molecular basis of how plants sense and adapt to the stress of zinc deficiency. We suggest that the characteristic His/Cys-rich domains in the bZIP19 and bZIP23 proteins [2] might be involved in the direct sensing of zinc, (possibly via a conformational change) confering a zinc-sensor function to thes TFs [3]. In order to test this hypothesis, we will analyse the *in vitro* metal-binding properties of bZIP19/bZIP23 by SEC-ICP-MS (Size Exclusion Chromatography/Inductively Coupled Plasma Mass Spectrometry). Additionally, amino acid substitutions in bZIP19 and bZIP23, targeting the His/Cys-motifs, will also be analysed. The results obtained so far will be discussed.

METHODS

Cloning of bZIP19 and bZIP23

For protein expression in Escherichia coli, forward primers of bZIP19 and bZIP23 contained an XmnI restriction site and reverse primers containing HindIII site were designed. bZIP19 and nZIP23 flanked by XmnI/HindIII were subcloned into pMAL-c2 for N-terminal protein fusion to maltose-binding protein (MBP; New England Biolabs). Proofreading PCRs were performed by LA Taq (Takara Bio). PCR products were verified by sequencing.

Protein expression and purification

The pMAL-c2 vector containing N-terminal fusion of bZIP19 or bZIP23 to MBP were expressed in *E. coli* strain BL21(DE3) and BL21(DE3)PlyS (Novagen) by standard procedures.

RESULTS AND DISCUSSION

We propose a model of a putative function as zinc-sensor for bZIP19 and bZIP23 transcription factors (TFs) (Figure 1). In order to test this hypothesis the *in vitro* metal-binding properties of bZIP19/bZIP23 will be analysed by SEC-ICP-MS (Size Exclusion Chromatography/Inductively Coupled Plasma Mass Spectrometry). As a starting point we are optimizing the bZIP19 and bZIP23 protein expression and purification protocol. According to the expected protein molecular weight we were able to express the recombinant bZIP-MBP fusion protein (Figure 2).

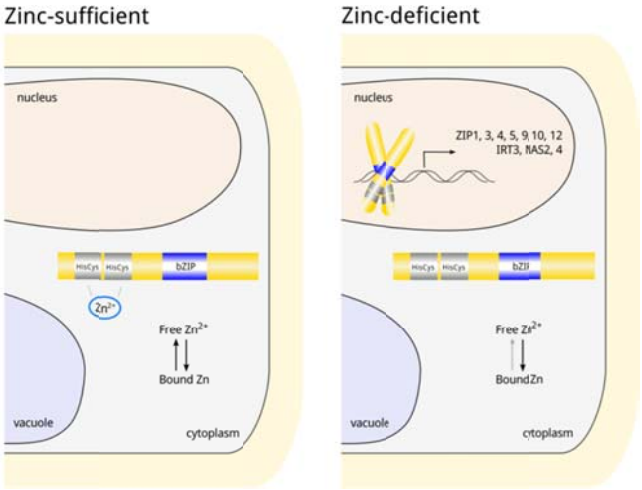

Figure 1. Schematic model of a putative function as zinc-sensor for bZIP19 and bZIP23 transcription factors (TFs).

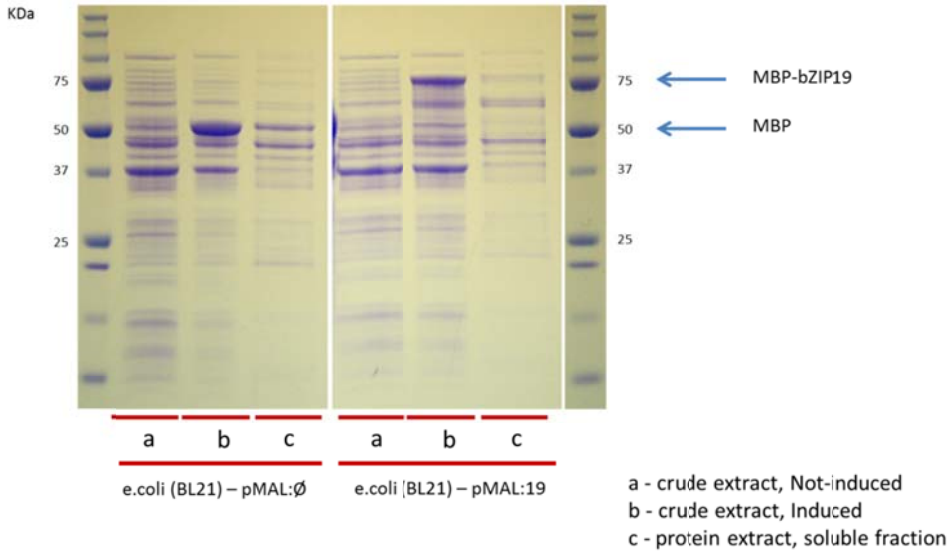

Figure 2. PageBlue protein stains of recombinant MBP-bZIP19 protein separated by SDS-PAGE.

CONCLUSIONS

We were able to express the recombinant bZIP-MBP fusion protein. The confirmation of protein identity, cleavage of bZIP from MBP, and protein concentration and purification are ongoing work.

ACKNOWLEDGEMENTS

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Selenium Concentration in Sheep's Blood and Animal Feed in Kosovo

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ABSTRACT

Preliminary studies of soil, cereal and animal feed samples from former Yugoslavia indicated low selenium (Se) level concentrations. In Kosovo no such study has previously been conducted. Therefore this study was conducted to assess the concentration of Se in sheep and the supplied feed. The survey included 294 sheep (152 blood samples from ewes, 132 samples from lambs and 10 samples from rams) and 66 different feed samples from 30 farms in 6 different districts of Kosovo. Ewes were of two years age and lamb of 1-2 months age. Feed samples differed based on what farmers were using to feed their sheep on the time of collecting samples. This survey showed inadequate (deficient or marginal) level of Se in both sheep blood and feed samples. In about 30 % of ewes and lambs the level of Se was under 0.05 µg/ml of blood (under this level clinical signs of White muscle Disease can be seen in lambs). Nearly 83 % of the feed samples contained < 0.05 µg/g Se in feed, whereas the optimal level is 0.1µg/g Se. A significant difference in Se levels of blood and feed samples among farms was observed. However, the significant difference, between districts was observed only in blood samples.

Keywords: Selenium, lambs and sheep, deficiency, animals feed

INTRODUCTION

Selenium is a trace but very important chemical element for human and animal nutrition. Selenium is considered to be deficient in most of the countries in the world, but in some countries selenium toxicity was observed. Two are the main sources of toxicity: selenium accumulator plants in seleniferous soils or high amounts of selenium given as supplement in feed or given by injection. Selenium toxicity and deficiency limits are very narrow. In Balkan countries as well as in most of European countries selenium was found to be in deficient levels.

Selenium is integral part of many enzymes and its main effect is as antioxidant. Selenium deficiency is related with many of animal pathologies or diseases like: muscular dystrophy, White Muscle Disease, retained placenta, immune response etc. In humans the most known diseases are Keshan Disease (a congestive cardiomyopathy) and Kashin Back Disease (endemic osteochondropathy). Also selenium deficiency is supposed to be related with endemic nephropathy and the urinary tract tumors.

MATERIALS AND METHODS

Blood and feed samples were collected from 30 farms in 6 different districts of Kosovo. Blood samples were collected from ewes (in 30 farms), lambs (in 27 farms) and rams (in 5 farms). Blood samples are collected from jugular vein in vacutainer tubes with heparin and saved in deep freeze conditions. At the same time 60 feed samples are collected from the same farms (2-3 feed samples in each farm), depending on what farmers were using to feed their sheep on the time of collecting samples

Animal's whole blood was used for analysis. Samples were digested using microwave assisted nitric acid decomposition (UltraClave, Milestone). Total Selenium concentration was measured using inductive coupled plasma mass spectrometry (ICP-MC) (Agilent Technologies 8800 series).

RESULTS AND DISCUSSIONS

The significant variation in blood selenium concentration was observed between different farms and between different districts, but this variation was not significant between farms fed without any kind of selenium supplement. A significant difference on selenium concentration was observed between animals fed without selenium supplement and those supplied with any kind of it (feed compound, mineral stone or injectable selenium). A significant correlation was observed between blood and feed selenium concentration within the same farms. No significant variation was observed between adult sheep (ewes) and lambs.

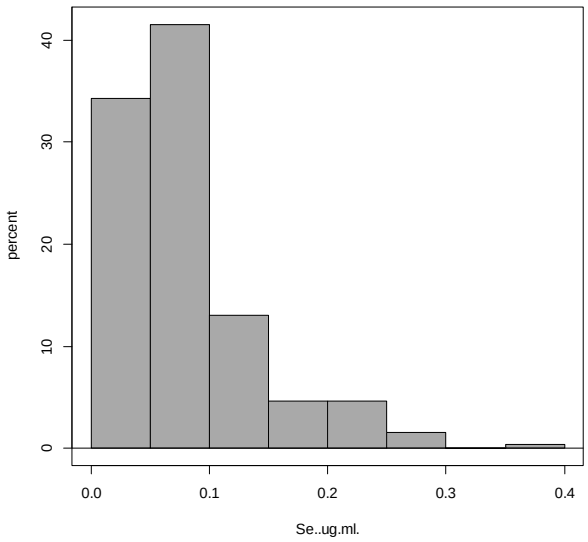

Figure 1. Percentage distribution of selenium concentration in sheep

CONCLUSIONS

From this study we can conclude that selenium is in deficient or marginal level in almost all sheep feed without any kind of selenium supplementation. Therefore selenium should be supplemented to animal feed either with premixes in feed compound, by mineral stones or by injectable selenium solutions.

Selenium Composition of Commercial Beers and its Retention During the Brewing Process

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INTRODUCTION

Suboptimal intake of Se is associated with increased risk of physiological dysfunction in humans (Hart *et al*., 2011). Dietary intakes based on ~1 µg Se kg^{-1} of body weight day^{-1} are recommended in the UK by the Committee on the Medical Aspects of Food Policy (COMA); however, reported intake ranges between only between 48 and 58 µg day^{-1} (Hart *et al*., 2011) According to the FAO (FAO, 2011), UK beer consumption achieve 78 kg beer $capita^{-1}$ $year^{-1}$. This beverage, is an important source of nutrients such as vitamins, carbohydrates, magnesium, potassium, folic acid and polyphenols among others, it could represent a source of Se in human diet, although detailed Se composition data are lacking in the literature. Furthermore, while there is information about Se retention during the manufacture of cereal derived foods such as bread in the literature, there is a lack of information regarding Se retention during the brewing process. The aims of this study were (1) to determine the likely contribution of beer to dietary Se intakes based on a range of commercial beers and (2) to determine the retention of Se during brewing from grain to final product.

METHODS

The Se composition of 128 beers, representing different brands and styles was determined. The survey included products designated as ales (n=68, defined as beer with top fermentation), lagers (n=60, defined as beer with bottom fermentation) and lambics (n=4, defined as beer fermented with wild yeasts) from 16 different countries/areas in total. Samples were diluted 1-in-6 with 1% HNO_3 and analysed using inductively coupled plasma mass spectrometry (ICPMS) using hydrogen reaction cell (X-SeriesII, Thermo Fisher Sciencific Inc., Waltham, MA, USA).

Malting and brewing trials with ^{77}Se enriched wheat and ^{77}Se analysis:

Fifteen ^{77}Se enriched wheat samples (250 g) coming from 3 different fields (which had received 10 g Se ha^{-1} as isotoptically enriched $Na_2{}^{77}SeO_4$) were malted with a steeping cycle of 9 h wet, 15 h dry, 9 h wet, 15 h dry, 2.5 h wet at 16 ºC. Germination and kilning were done as follows: Germination= temperature 19 ºC for 3.5 days; Kilning: 55 ºC for 16 h, 72 ºC for 4 h. Roots and sprouts were removed manually. Milled grist (1/1 barley/wheat mixture) was mashed and fermentated with *Saccharomyces cerevisiae* in 100ml glass serum bottles fitted with air-tight rubber septa to maintain an anaerobic an environment. Samples were digested under microwave (Multiwave 3000, Anton Paar GmbH) heating for 45 min at a controlled pressure of 20 bar in 3.0 mL of 50% trace analysis grade HNO_3, 2.0 mL 30% H_2O_2 and 3.0 mL milli-Q water and then diluted to 20 mL with milli-Q water. Immediately prior to analysis, samples were re-diluted 1-in-10 (solid samples) or 1-in-7.25 (liquid samples). ^{77}Se analysis was undertaken using ICP-MS using a hydrogen reaction cell.

RESULTS AND DISCUSSION

The Se composition of a range of commercial beers

The Se concentration of beers from the USA and Canada , are on average higher (> 10 µg Se L^{-1}) than the one obtained in the rest of the beer brewed in other areas (Table 2). Such differences are likely to be due to higher concentration of Se in grains grown in the USA and Canada than in Europe, (Broadley *et al*., 2006).

The retention of Se during brewing

The major losses of Se during brewing are in the mashing step (around 54%), where ^{77}Se is wasted with the mash residue. Steeping process cause the loose of aprox. 20% of the ^{77}Se, by dilution in the steeping water. Last important ^{77}Se loose roots is observed after germination, kilning and roots removal (about 14%); this could be due to the ^{77}Se phytovolatilization through ^{77}Se transformation into, dimethylselenide (DMSe) or dimethyldiselenide (DMDSe) (Grant *et al*., 2004).

Table 1. Mean Se concentrations (µg L^{-1}) and range of all beers included in the survey by countries/areas

Origin country	Selenium content (µg L^{-1})	Range (µg L^{-1})	Origin country	Selenium content (µg L^{-1})	Range (µg L^{-1})
Argentina	3.08		Holland	1.42	0.79-2.01
Belgium	1.66	0.7-307	Ireland	1.43	0.65-2.59
Canada	17.53		Italy	1.87	1.66-2
China	5.85	5.37-6.58	Mexico	3.53	0.82-8.06
Czech Republic	1.25	0.84-1.41	Russia	2.01	
England	1.89	0.66-5.54	Scotland	1.27	0.86-2.41
EU	0.96	0.88-1.1	UK	1.57	0.59-4.77
Germany	0.92	0.73-1.68	USA	10.97	2.9-20.15

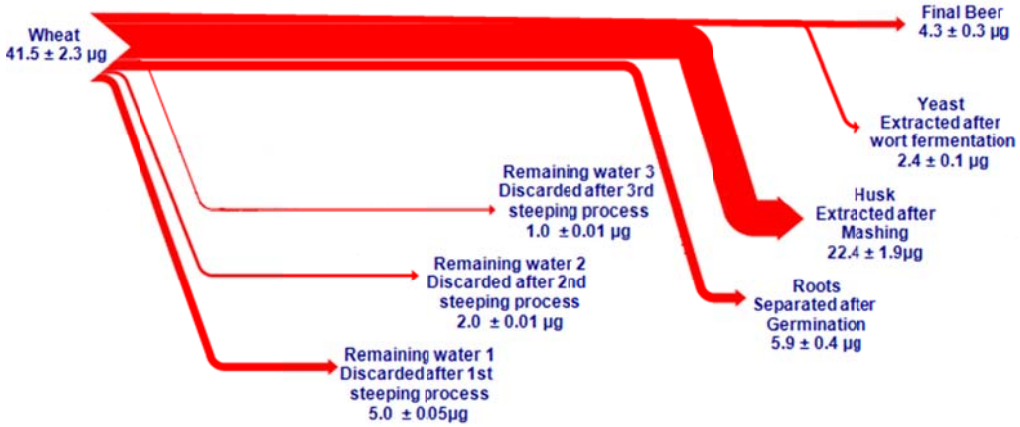

Fig. 1: A mass balance of Se retention during the brewing process from wheat grain to beer. The Se mass is based on a sample of 250 g of grain. Note, the mass components of each fraction do not add up exactly to the starting mass as each component was measured directly.

CONCLUSIONS

The Se concentration in commercial beer depends on the origin country, and is likely to be due to the plant-available concentration of Se in soils. Around 10% of the Se in the cereal grain is transferred into fermented beer with the highest losses occurring during the mashing step.

ACKNOWLEDGEMENTS

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Speciation of Selenium in Mature Grains of Wheats Grown Under Selenium-Supplementation Regimes in Actual Field Conditions

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INTRODUCTION

Agronomic biofortification of staple crops is an effective way to enhance their contents in essential nutrients and make them available up the food chain, with a view to correcting for their deficiencies in animals or humans. Selenium (Se) is one such case, for its uneven distribution in the continental crust and, therefore, in agricultural lands easily translates into substantial variation in dietary intake and nutritional status. Cereals are far from being the main sources of Se on a content basis, but they are likely the major contributors to intake on a dietary basis.

METHODS

To assess Portuguese wheat potential to assimilate and biotransform Se, bread (*Triticum aestivum* L.; Jordão cultivar) and durum (*Triticum durum* Desf.; Marialva cultivar) wheats were enriched with Se through foliar and soil addition at an equivalent field rate of 100 g of Se per hectare (ha), using sodium selenate and sodium selenite as Se-supplementation matrices, in actual field conditions throughout. Biotransformation of inorganic Se was evaluated by using HPLC–ICP-MS, after enzymatic hydrolysis for Se-species extraction in the resulting mature wheat grains. The speciation procedure has been validated through analyses of a Se-enriched yeast, certified reference material (SELM-1).

RESULTS AND DISCUSSION

All analyzed samples – blank and supplemented ones – showed that, regardless of scale magnitude, selenomethionine (SeMet) was invariably the major Se species found in wheat grains. Other minor Se species were found as well: one peak around 2 min may correspond to selenocysteine ($SeCys_2$) or selenomethionine Se-oxide (SeMetO), or to a combination of both, since that first peak cannot be unambiguously assigned to $SeCys_2$. The last peak, around 9 min of elution time, corresponds to Se^{VI}.

SeMet was the major species found in all samples, where 70 % to 100 % (Table 1) of their total Se is in the form of SeMet. The results also show that, regardless of the chemical form of inorganic Se used in the supplementation (Se^{IV} or Se^{VI}) and the supplementation procedure itself, conversion to SeMet was almost complete. The highest value of Se^{VI} (11 %; Table 1) was found in samples with top levels of total Se, suggesting that Se supplementation to wheat plants at very high rates can result in higher concentrations of inorganic Se in the form of Se^{VI}. The presence of KI as an additive (for a side study) does not seem to affect the total accumulation of Se in mature grains or the distribution of selenocompounds. Wheat flours from grains devoid of any type of supplementation were analyzed as well. When compared to supplemented ones, these samples show much lower concentrations of total Se and also feature SeMet as the major species, pointing to a most likely association between total Se and SeMet.

Table 1. Se-species' percentages in mature grains by ICP-MS. Selenium supplements: 100 g Se ha^{-1} as sodium selenite (Se^{IV}) or sodium selenate (Se^{IV}), plus 10 μM of KI per plot where applicable.

	SeMet (%)	Se^{VI} (%)		SeMet (%)	Se^{VI} (%)
Durum wheat			**Bread Wheat**		
Foliar application					
(3) Blank	73±1	2.5±1.1	(3) Blank	75±8	–
Booting					
(3) Na_2SeO_3	58±20	3.6±1.1	(3) Na_2SeO_3	86±13	–
(1) Na_2SeO_3+KI	100	2	(3) Na_2SeO_4	61±4	3.2±0.7
(3) Na_2SeO_4	60±8	3.6±1.1			
(3) Na_2SeO_4+KI	54±4	1.8±1.2			
Grain filling					
(2) Na_2SeO_3	69±2	5.1±2.1	(3) Na_2SeO_3	63±9	–
(3) Na_2SeO_3+KI	63±2	–	(3) Na_2SeO_4	63±8	0.4±0.1
(2) Na_2SeO_4	68±11	11.1±0.1			
(3) Na_2SeO_4+KI	65±6	5.0±0.6			
Soil addition					
(2) Blank	100	3.4±4.9	(1) Blank	65	3.1
(2) Na_2SeO_4	95±17	0.5±0.4	(3) Na_2SeO_4+KI	86±4	1.1±0.3
(2) Na_2SeO_4+KI	72±16	0.9±0.1			

CONCLUSIONS

Regardless of the chemical vehicle of Se used in the field supplementation – selenite or selenate, that is Se^{IV} or Se^{VI} as active forms – and of the supplementation procedure itself, the major species in all samples was SeMet, meaning that the conversion of inorganic Se to SeMet was almost complete. Higher levels of total Se resulted in higher SeMet concentrations, suggesting that both wheat varieties are likely to assimilate SeMet in mature grains proportionally to the corresponding field supplementation rate. The results also show that an agronomic biofortification of wheat crops with Se can improve the nutritional quality of mature wheat grains by boosting their levels of SeMet, and thus providing an attractive option for enhancing the Se status in human populations with Se-deprived diets through Se-enriched, wheat-based foodstuff.

ACKNOWLEDGEMENTS

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Poster Presentations

Phyto-Mediated Biostimulation of the Autochthonous Microbial Community for the Depletion of Polycyclic Aromatic Hydrocarbons in Contaminated Sediments

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic contaminants causing significant hazards to many organisms including humans. The main objective of the current study was to validate the vegetation of dredged sediments with the highly transpiring plant *Phragmites australis* as an exploitable biostimulation approach to accelerate the depletion of PAHs. The vegetation with *P. australis* was an efficient biostimulation approach for the depletion of an aged PAH contamination (229.67 ± 15.56 µg PAHs/g DW) in dredged sediments. *P. australis* plants promoted the oxidation of the PAHs, comprising the high molecular fraction, by rhizodegradation. Quantitative real time-PCR reactions have been performed and the results indicated that Gram positive (GP) degraders resulted to be selectively favored by vegetation with *P. australis*. The metabolic activity of the GP degraders resulted to be mandatory for the depletion of the high molecular weight and recalcitrant six condensed rings indeno[1,2,3-cd]pyrene and benzo[g,h,i]perylene.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a large group of organic contaminants deriving principally from anthropogenic sources. Due to their low water solubility, high hydrophobicity, complex chemical structure, recalcitrance to biodegradation, PAHs tend to accumulate in the soil and sediment organic matter. Among biostimulation approaches, vegetation with plant species found applications for accelerating the depletion of disparate types of very recalcitrant contaminants including PAHs (Di Gregorio et al., 2013). The objective of the current study is the validation of the vegetation of dredged sediments with *P. australis* as an exploitable biostimulation approach to accelerate the depletion of PAHs. In addition we quantified the levels of transcription of the bacterial Gram positive (GP) and Gram negative (GN) PAH-RHDα genes encoding for the enzymes priming the PAH oxidation.

METHODS

A total of 42 replicates containing 25 kg of air-dried dredged sediments were prepared in plastic pots and maintained at 24±1 °C in controlled growth chamber. A 36 pots were spiked with NH_4NO_3 dissolved in the saturating water to reach a C:N ratio of 10:1. The other 6 pots not spiked with N (not biostimulated) were analyzed after 12 months of incubation as controls. A total of 18 N spiked pots were vegetated each with 5 *P. australis* plants/pot. After 6, 9 and 12 months of incubation, 6 vegetated pots for each time point were processed for chemical analysis. Sediments and plant samples were collected and analyzed for PAH content. Identity of the PAHs was confirmed by the retention time and abundance of quantification/confirmation ions using GC/MS. The total RNA from sediments has been purified using the MoBio RNA power soil kit. Quantitative real-time PCR reactions were carried out on an ABI Prism® 7300 Sequence Detection System using Sybr® Green PCR Master Mix.

RESULTS AND DISCUSSION

The results show that after 12 months of incubation, no PAH depletion was observed in the not biostimulated sediments (notB) (Table 1). On the other hand, either in N spiked (Ns) and in N spiked and vegetated sediments (NsV) the PAH depletion has been recorded (Table 1). In fact, biostimulation induced the progressive depletion of the different PAHs that resulted to be more consistent in the case of vegetation with *P. australis* plant. In N spiked sediments the depletion of the 16 PAHs occurred mainly during the last three months of incubation, after a lag phase of nine months. Moreover, the six ring condensed Indeno[1,2,3-cd]pyrene (PI) and Benzo[g,h,i]perylene (BP) were not depleted, even after 12 months of incubations. On the other hand, in N spiked and vegetated sediments a gradual depletion of all the different PAHs has been observed. After 12 months of incubation, the depletion of naphthalene accounted for the ca 80% in N spiked and vegetated sediment and for 46% in N spiked and not-vegetated sediments. With reference to the 3 condensed ring PAHs the % of depletion varied between 60% and 80% with a mean value of 68% in N spiked and vegetated sediments. In N spiked and not-vegetated sediments the % of the 3 condensed ring PAHs depletion varied between 29% and 38% with a mean value of 34% (Table 1).

In relation to the vegetated sediments, results obtained indicated a stronger effect of the vegetation with *P. australis* on the metabolically active microbial population. After 6 months, significant increases in the fractional copy number of the GN PAH-RHDα transcripts have been observed both in the rhizospheric and the bulk portion of vegetated sediments. Higher values reached in N spiked sediments, 36 in rhizospheric and 41 in bulk portion of the vegetated sediments, with reference to the beginning of the experimentation. Interestingly remarkable increments in the fractional copy number of the GP PAH-RHDα transcripts have been observed in the rhizospheric portion of the vegetated sediments ($P<0.05$). After 6 months, the corresponding values were 44.8 higher than the one at the beginning of the experimentation, increment that exceeded the one of the GN PAH-RHDα transcripts. The N amendment restored the nutritional conditions that allowed the autochthonous microbial population competent for the PAH oxidation to reach a density of metabolic active candidates sufficient to initiate a process of natural attenuation of the PAH contamination. The GN degraders played a pivotal role in the process, however, no

depletion of the 6 condensed ring PAHs has been recorded in N spiked sediments. On the other hand, in the rhizospheric portion of vegetated sediments the metabolically active GP degraders were numerically more represented than the GN one. The net increase in GP metabolically active degraders, observed only in vegetated sediments, positively correlated with the depletion of the 6 condensed ring PAHs, suggesting the involvement of these bacteria in the depletion of the high molecular weight PAHs.

Table 1. PAH concentration in sediment at the beginning (BE), 12 months of incubation of N-spiked sediments (Ns), 12 months of incubation of N-spiked and vegetated sediments (NsV), 12 months of control (notB).

PAH	**Abbr**	**BE (mg/Kg DW)**	**NsV (mg/Kg DW)**	**%**	**Ns (mg/Kg DW)**	**%**	**notB (mg/Kg DW)**	**%**
2condensed rings				80		46		ns
Naphthalene	NA	19.0 ± 1.0	3.8 ± 0.1		10.3 ± 0.1		18.9 ± 0.1	
3condensed rings				68		34		
Acenaphthylene	ACE	14.7 ± 2.0	5.9 ± 0.1		9.9 ± 0.3		14.6 ± 0.3	
Acenaphthene	AC	33.9 ± 1.0	6.8 ± 0.1		21.9 ± 1.1		33.2 ± 0.2	
Fluorene	FL	19.8 ± 1.2	6.2 ± 0.1		12.5 ± 0.7		19.7 ± 1.0	
Phenanthrene	PH	19.2 ± 0.2	6.0 ± 0.2		11.9 ± 0.2		19.1 ± 0.1	
Anthracene	AN	15.0 ± 2.5	5.4 ± 0.1		10.6 ± 0.2		15.0 ± 0.1	
4condensed rings				51		34		ns
Fluoranthene	FLU	15.6 ± 2.1	4.9 ± 0.3		9.0 ± 0.2		15.6 ± 0.1	
Pyrene	PY	12.9 ± 1.7	7.0 ± 0.1		8.0 ± 0.1		12.7 ± 0.1	
Benzo[a]anthracene	BaA	14.9 ± 0.7	9.0 ± 0.1		12.0 ± 0.2		14.8 ± 1.1	
Chrysene	CH	13.9 ± 0.2	7.0 ± 0.2		9.0 ± 0.1		13.6 ± 0.4	
5condensed rings				37		24		ns
Benzo[b]fluoranthene	BbF	12.2 ± 0.1	7.9 ± 0.1		10.7 ± 0.1		12.1 ± 0.1	
Benzo[k]fluoranthene	BkF	7.9 ± 0.1	5.0 ± 0.1		5.0 ± 0.1		7.8 ± 0.1	
Benzo[a]pyrene	BaP	9.0 ± 0.1	5.4 ± 0.1		6.9 ± 0.1		8.9 ± 0.1	
Dibenz[a,h]anthracene	DA	8.0 ± 0.3	5.2 ± 0.1		6.2 ± 0.1		7.9 ± 0.2	
6condensed rings				30		ns		ns
Indeno[1,2,3-cd]pyrene	PI	6.9 ± 1.2	4.9 ± 0.1	30	6.4 ± 0.1	ns	6.7 ± 0.1	
Benzo[g,h,i]perylene	BP	7.0 ± 1.0	4.9 ± 0.1	30	6.4 ± 0.1	ns	6.9 ± 0.1	
∑ PAHs		229.7 ± 15.6	95.4 ± 1.6	59	156.67 ± 3.5	32	227.4 ± 4.2	ns

ns, not significant (P>0.005)

CONCLUSIONS

The phyto-based biostimulation of the PAH depletion in dredged sediments resulted to be successful. A reduction of 59% of PAHs has been observed after one year of treatment compared to the 32% in the not vegetated sediments. The *P. australis* was actually capable to favor the metabolism of both GN and GP PAH degraders in the plant associated microbial community as well as in the bulk portion of the vegetated sediments. However, the GP degraders were significantly favored. Since the metabolic activity of the GP degraders resulted to be mandatory for the depletion of the six condensed rings PAHs, the exploitation of *P. australis* resulted to be significantly beneficial.

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Use of Radial Differentiation Coefficient in Assessment of Contaminant Behaviour in Soil Profile

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INTRODUCTION

A plant in the soil–plant system is the biogeochemical barrier for contaminants in soil. The biogeochemical barrier is one of soil barriers that determines contaminant migration patterns and extent and thus have impact on contaminant fate in soil. This barrier is based on the concentration function of plants which is highly displayed under the conditions of technogenic contamination in association with a soil profile. In soil profile contaminants actively redistribute over the time and especially under the conditions of technogenic contamination.

To describe the quantitative differentiation of contaminants in a soil profile, radial differentiation coefficient is used and example of heavy metal accumulation/leaching over the soil profile in the territory under the technogenic contamination (oil refinery) is presented.

METHODS

Radial differentiation coefficient K_{rd}^{i} shows the accumulation level of a heavy metal in parent rock of the respective soil horizon (Lietuvninkas 2012) and is calculated by the Equation:

$$K_{\mathrm{rd}}^{i} = \frac{C_{\mathrm{dir},j}^{i}}{C_{\mathrm{uol}}^{i}},$$

where $C_{\mathrm{dir},j}^{i}$ is the mean concentration of heavy metal *i* in *j*-th soil horizon, mg/kg DW, C_{uol}^{i} is the concentration of heavy metal *i* in soil-forming rock, mg/kg DW.

RESULTS AND DISCUSSION

The radial differentiation of heavy metals in the soil profile at the depth of 0–10, 10–30 and 80–100 cm is presented in Fig. When $K_{\mathrm{rd}}^{i} > 1$, it signifies heavy metal accumulation. Otherwise, when $K_{\mathrm{rd}}^{i} < 1$, it is indicative of heavy metal washout in the respective layer of soil. In this respect, heavy metals analysed in the study may be divided into two groups based on their behaviour. The first group comprised heavy metals which were accumulated in the soil of the area of the refinery influence (Zn, Ni, Pb and Cr) and the second group consisted of metals washed out from the soil (Cu) (Fig.).

Based on heavy metal accumulation in the soil, heavy metals may be arranged in descending order as follows: Cr>Pb>Ni>Zn>Cu (Fig.) (Baltrėnaitė et al. 2014).

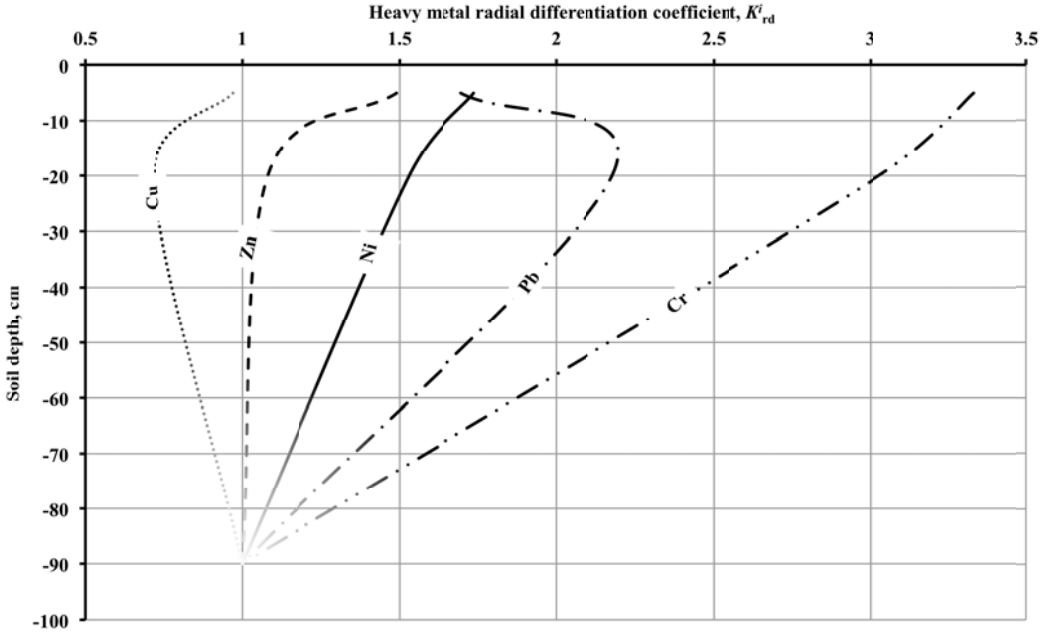

Fig: The variation of radial differentiation coefficient (K^i_{rd}) values for heavy metals in the soil profile of the area of AB ORLEN Lietuva influence

CONCLUSIONS

The radial differentiation coefficent indicated that accumulation pattern of heavy metals the soil in the territory under influence of aerogenic technogenic contamination (oil refinery) followed the descending order: Cr>Pb>Ni>Zn. The highest values of the coefficient were characteristic of typomorphic contaminant related to a contamination source in question.

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Assessing Zinc Content in Brazilian Cassava for Biofortification Purposes

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INTRODUCTION

Zinc (Zn) deficiency is a well-known health problem in humans, especially in populations from developing countries. Biofortification is a strategy that aims to increase the content of selected micronutrients, including Zn, in staple foods. Cassava (*Manihot esculenta* Crantz) is a staple crop of tropical and subtropical developing countries, feeding some 600 million people a day worldwide. The nutritional quality of the cassava root is not sufficient to meet all dietary needs and more studies on biofortification of this crop can lead to an improvement of its nutritional quality. This study evaluated Zn contents in cassava accessions of a Cassava Active Germplasm Bank.

METHODS

The samples were collected from the Active Cassava Germplasm Bank of Embrapa Cerrados. The area was previously fertilized with 300 kg ha^{-1} of the commercial fertilizer NPK 4-30-16 + Zn applied at the start of the growing season. Additional cultural practices were applied following the recommendation for cultivating cassava. The Germplasm Bank was conducted in field plots of 1.20 m x 0.80 m with 10 plants in each line.

We first collected leaves from 464 cassava accessions and evaluated the Zn content in them. Next, we choose the accessions with high and low Zn contents to evaluate Zn content on the roots (n=53).

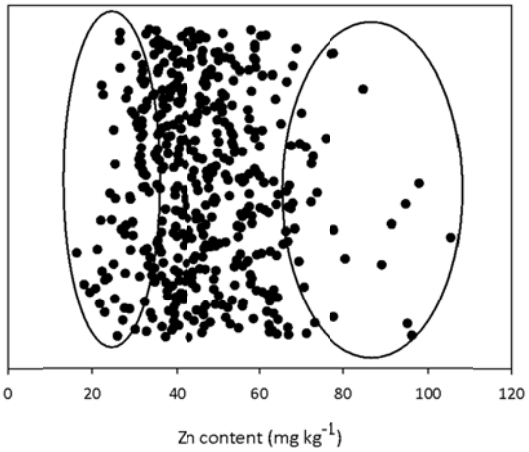

Fig. 1 – Zn content in Cassava leaves (n=464). Dots inside the circles were selected for further Zn analysis in roots.

For Zn analyses, the samples were microwave digested according to U.S. Environmental Protection Agency Method 3051A (USEPA, 1998). The Zn contents in the digested solutions were determined by flame atomic absorption spectrometry. Standard reference materials from the National Institute of Standards & Technology – SRM 1573 Tomato leaves – were used to substantiate the accuracy of the analytical results obtained. All results are expressed as the means of three replicates. The treatment effects were determined by an ANOVA and Scott-Knott's test to investigate statistically significant differences at $p \leq 0.05$.

RESULTS AND DISCUSSION

The mean Zn concentrations of the repeated analysis (n=5) of the standard reference was 28.1 mg kg^{-1} (certified value: 30.9±0.7 mg kg^{-1}). The Zn content recovery in the certified samples (91%) showed a reliable analytical data accuracy for Zn analysis.

The Zn content showed over 3-fold variation between accessions with high and low Zn content, ranging from 4.9 to 15.8 mg kg^{-1} dry weight (DW) (Figure 2). The highest values were found in the accessions BGMC 593 e BGMC 1451 (15.3 and 15.8 mg kg^{-1}, respectively).

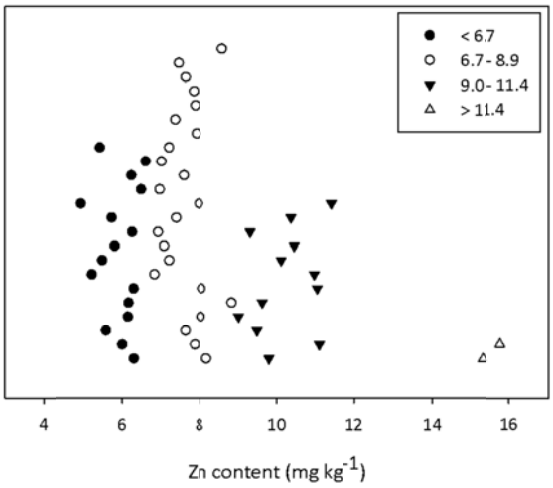

Fig. 2 – Zn content in roots of Cassava. Accessions with different symbols are significantly different.

Earlier investigations have found significant genotype variation in Zn content in cassava roots (Chávez et al., 2005; Mezette et al., 2009). Such differences may be related to physiological and morphological characteristics (Morishita et al., 1987).

CONCLUSIONS

Our results suggest that Zn content in cassava accessions are strongly influenced by genetic variability. Thus, there is a great potential to explore the Brazilian cassava germplasm in order to increase the Zn content for biofortification purposes using genetic strategies.

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Effect of Different Fertilization Strategies on the Zinc Content of Wheat and Maize

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INTRODUCTION

Zinc (Zn) is an essential micronutrient for all living organisms. It has structural and functional roles in metalloenzymes, biomembranes, transcription processes and gene expression. The first cause of Zn deficiency in human nutrition is inadequate dietary intake of bioavailable Zn (Hotz et al., 2004). Populations living in regions where the daily calorific intake is mostly based on cereals are particularly exposed to Zn deficiency, as plant-based diets have generally low Zn contents and enclose antinutrients. Increasing the Zn content in the edible part of crops might contribute to alleviate the problem of Zn deficiency in human nutrition.

Low Zn content in crops can be linked to two major causes: i) low Zn content of the soil and ii) low phytoavailable Zn in the soil. The present project is investigating the impact of fertilization strategies, with emphasis on organic fertilizers, on the Zn transfer from the soil to the plant. On the one hand organic fertilizers can be considered as an important Zn source (direct effect), and on the other they can change the phytoavailability of zinc by changing the physicochemical and biological properties of the soil (indirect effect).

The objective of is study is to determine the effect of different organic fertilizer inputs on the phytoavailability of Zn in agricultural soils. Therefore three long-term field trials with different fertilization strategies have been selected to highlight the effect of organic fertilizer on the zinc content in wheat and maize.

METHODS

The Zn status of long-term field experiments with contrasting fertilization strategies and soil properties especially regarding pH will be compared. Three sites have been chosen: i) the “DOK” which was set up in 1978 (Therwil near Basel, Switzerland) (Mader et al., 2002), the “ZOFE” which was established in 1949 (Zurich-Reckenholz, Switzerland) (Walther et al., 2001) and the “MASCOT” field trial set up in 2001 (Pisa, Italy) (Mazzoncini et al., 2010). Whereas DOK and ZOFE have slightly acidic soil conditions, the soil pH from MASCOT is alkaline. The applied organic fertilizers are specific to each field trial and are covering the most common organic fertilizers used in agriculture: manure, compost, sewage sludge, dry manure pellets, dry bovine blood but also mineral fertilizer, all added in agronomically relevant quantities. No mineral Zn fertilizer was added in any of these long-term field trials. In DOK and MASCOT total Zn content of wheat grain and straw from the harvest 2012 was measured. In ZOFE the same measurements were realized on maize grain from the harvest 2012. Soil Zn analyses were also considered in this study. The plant available Zn is estimated by diethylene triamine pentaacetic acid (DTPA) extraction after Lindsay and Norvell (1978). Total Zn of the soil and of the biomass was analyzed by X-ray fluorescence spectrometry (XRF).

RESULTS AND DISCUSSION

The results suggest that the Zn concentrations of the biomass in all long-term field trials is inversely proportional to the yield, even though the homeostasis of the plant seems to limit strong variations. The Zn concentration of the biomass does not reflect the variations of DTPA-extractable Zn of the soil. The total Zn export from the field compared to the data of the DTPA-extractable Zn from the soil suggest that organic fertilizers directly or indirectly help to refill the phytoavailable Zn-pool in the soil. This is not the case in the mineral fertilizer treatments.

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Influence of Different Peach Rootstocks on Leaf Mineral Content in cv. 'Redhaven' (*Prunus persica* (L.) Batsch.) in relation to the Yield and Fruit Quality

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INTRODUCTION

Plant growth and fertility mostly depend on pedoclimatic conditions. For good economic production success it is very important to consider optimal grown conditions especially for perennial plants, such as peach trees. Peach tree is very sensible, if it is planted on replant soil with the same rootstock as it was planted before, otherwise it comes to soil exhaustion leading to physiological problems on trees and consequently reflects on lower yield and fruit quality (Loreti and Massai, 2002).

Peach orchards in Slovenia are mostly planted on replant soil, therefore it is important to know, which is the best rootstock for our environmental conditions that resists against pathogens in the soil and achieves the optimal growth. In Fruit Growing Centre Bilje different rootstocks with annually observing their vigor, fertility, fruit quality and vitality were tested. We try to obtain good tree fertility status with applying all necessary agricultural measures (pruning, irrigation, pest control and treatments,…) and also appropriate soil and foliar nutrition. A real 'picture' of tree nutrient status only soil and leaf analyses can represent, that is why we carried out soil analyses before planting the trees and three years later and leaf analyses also to check, if several rootstocks influence to nutrient uptake.

In this paper we present the leaf nutrition status in 'Redhaven' cultivar on eleven different rootstocks on replant soil in the six year in relation to fertility and fruit quality in the first four years lasting period.

METHODS

In 2005 11 different rootstocks: Cadaman, Adesoto 101, Barrier 1, Isthara, Julior, Monegro, MrS 2/5, Tetra, Penta, peach seedling and GF 677 (as a standard) were grafted with cv. 'Redhaven' and planted in Fruit Growing Centre of Bilje. During growing seasons we took care for optimal growth with applying all necessary measures. In 2011 we analyzed leaves 116 days after full bloom (Heckman, 2004; Montanes and Sanz, 1994) in order to check nutrient status in the trees.

Leaves were analysed according to ISO 6869:2001: 1 g of dry sample was incinerated at 550 °C and diluted in 5ml of HCl (6M) and diluted to 100 ml. K, Ca, Mg, Fe, Zn, Mn and Cu have been analysed with atomic absorbtion (Varian AA55), while P according to ISO 6491 on spectrophotometer and N according to ISO 5983.

Statistical evaluation of data has been done with Statgraphic Plus, 4.1.

RESULTS AND DISCUSSION

As expected, results of foliar analyses (Table 1) indicate good macronutrients status of all plants, although there are surprisingly high values of calcium, especially on Monegro (higher than optimal range according to Bergmann, (1992). There are statistically significant differences between rootstocks:

- significantly higher contents of potassium on Penta and Adesoto rootstock than in the standard rootstock GF 677
- significantly higher contents of magnesium on Barrier 1 rootstock and lower on Tetra, Penta, Adesoto and MrS 2/5 – all plum rootstocks as genetic origin

- ratio between K, Ca and Mg (K/ Ca + Mg) indicate statistically significant differences between rootstocks, similar as for individual macronutrients. Here we can expose Monegro rootstock with low ratio value and Tetra with high one.

Table 1: Leaf mineral content of 'Redhaven' cultivar on different rootstocks on replant soil in 2011.

Rootstock	N (g/kg)	P (g/kg)	K (g/kg)	Ca (g/kg)	Mg (g/kg)	K/(Ca+Mg) (g/kg)	Mn (mg/kg)	Zn (mg/kg)	Cu (mg/kg)	Fe (mg/kg)
Adezoto	35,0 ab	2,4 a	29,3 d	30,0 a	3,1 a	0,9 bcde	153,3 b	34,3 b	13,3 b	95,3 a
Barrier	34,7 ab	2,5 a	23,3 a	34,0 a	6,5 d	0,6 abc	80,7 a	25,0 ab	11,0 ab	82,0 a
Cadaman	35,3 ab	2,2 a	23,0 a	37,3 a	5,0 bcd	0,6 ab	115,3 ab	27,0 ab	12,0 ab	88,3 a
GF677	31,3 a	2,1 a	23,0 a	37,0 a	4,3 abc	0,6 abc	101,3 ab	22,3 ab	12,0 ab	83,3 a
Isthara	32,0 ab	2,0 a	24,7 abc	36,7 a	3,7 ab	0,6 abcd	83,0 a	25,0 ab	13,0 ab	96,0 a
Julior	34,7 ab	1,6 a	28,0 bcd	27,3 a	3,9 ab	0,9 cde	94,0 a	26,7 ab	12,3 ab	95,0 a
Monegro	31,7 a	2,2 a	22,7 a	53,3 b	5,1 bcd	0,4 a	115,7 ab	22,7 ab	12,3 ab	88,3 a
MRS2/5	36,0 ab	2,3 a	28,3 cd	31,0 a	3,2 a	0,8 bcde	147,0 b	33,0 b	13,3 b	91,0 a
Penta	37,7 b	2,4 a	31,0 d	29,7 a	3,1 a	1,0 de	129,7 ab	29,7 ab	13,7 b	89,3 a
P. seedling	34,0 ab	2,2 a	23,7 ab	30,3 a	5,6 cd	0,7 abcde	79,7 a	18,0 a	10,3 a	86,0 a
Tetra	32,7 ab	2,3 a	28,7 cd	27,0 a	2,8 a	1,0 e	88,7 a	25,7 ab	12,3 ab	83,7 a

Means in the same column followed by different letters are significantly different at $P < 0,05$ (HSD test).

The content of micronutrients also indicated good nutritional status of plants. Statistically non significant difference was demonstrated only in content of Fe.

Although nutritional status of all plants achieved values in optimal macro and micronutrients range (except for Ca), there are two rootstocks, MrS 2/5 and Isthara, that show better average yield in the period of 2008-2011 (Hudina et al., 2012). On fruit quality, measured in 2008 and 2009 we noticed high significance effect of years, rootstocks and their interaction on fruit weight, fruit firmness, soluble solids, total sugars, total organic acids and sugar/acid ratio (Orazem et al., 2011a and b).

CONCLUSIONS

Different rootstocks are one of the factors which significant influenced leaf mineral content in 'Redhaven' peach cultivar on replants soil; also the yield and fruit quality were influenced. We are in the final step of this experiment and we try to find the best correlation between rootstock, yield and fruit quality in order to keep adequately mineral nutrition status of the trees.

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Effects of Precropping and Residue Incorporation into Soil with low Zn Availability on Zn Accumulation by Two Wheat Cultivars

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INTRODUCTION

Using other agronomic methods than application of mineral fertilizers is a new strategy to biofortify cereal grains with Zn. In many countries, especially in arid and semi-arid regions, very little or no crop residues are left in the fields, as they are used for feeding animals or for fuel (Timsina and Connor, 2001). This practice, however, has the disadvantage that an important source of micronutrients is removed (Prasad, 1999) and that it does not make use of the potential of decomposing organic matter to increase the bioavailability of resident soil Zn for plant uptake by providing ligands and stimulating the activity of soil microorganisms. In calcareous soils, organic acids produced during crop residue decomposition may increase plant Zn uptake also by dissolving solid phases containing Zn (Singh et al., 2005). In this study we investigated the effect of the incorporation of clover, safflower, sunflower and sorghum precrop residues into a calcareous Zn-deficient soil on soluble soil Zn concentrations and on the uptake of Zn from this soil by two wheat genotypes.

MATERIALS AND METHODS

In alternative treatments, clover (*Trifolium pratense* L.) sunflower (*Helianthus annuus* L.), safflower (*Contamus tinectirus* L.), and sorghum (*Sorghum bicolor* L.) were grown in pots in the greenhouse of Isfahan University of Technology (IUT), Isfahan, Iran. The DTPA-extractable Zn concentration of the soil was 0.21 mg kg^{-1}. After harvest, the aboveground residues of these precrops were air-dried, chopped and incorporated back into the soil of the respective pots at a rate of 6.5 t DW ha^{-1}. In addition, a control treatment with no pre-cropping and residue application was set up. Fourteen days after incorporation of the residues, wheat was sown. The two cultivars used were Back Cross and Kavir, of which the first is more tolerant towards Zn-deficiency than the latter. Each cultivar/residue treatment was replicated 3 times. The plants were harvested after seven months at grain maturity. Soil, shoot and grain Zn concentrations were analyzed by means of atomic absorption spectrometry. Dissolved organic carbon (DOC) was determined in soil saturation extracts using a TOC analyzer.

RESULTS

All precrop/residue treatments increased the DTPA-extractable Zn in the soil compared to control treatment (Table 1). They had no significant effect on dry matter yield production. However, the safflower and clover treatments increased grain yield production in contrast to sorghum and sunflower (Table 1). The shoot Zn concentration of both cultivars increased in all precrop/residue treatments as compared to the respective control treatment. Incorporation of safflower and clover residues also increased the grain Zn concentration in both cultivars, whereas incorporation of sorghum and sunflower only increased grain Zn concentration in Back Cross and had no significant effect on grain Zn concentration in Kavir (Table 1).

In all residue treatments, Back Cross accumulated shoot and grain Zn at higher concentrations than Kavir (Table 1). There was a significant positive correlation between shoot and grain Zn concentration and DOC for Back Cross (Fig. 1), but not for Kavir.

DISCUSSION AND CONCLUSIONS

The results show that pre-cropping clover and safflower and incorporating their residues into soil can increase Zn bioavailability in calcareous Zn-deficient soil and thereby increase Zn accumulation in wheat, depending on the type of precrop and wheat cultivar. A mechanism possibly involved is mobilization of Zn through formation of organic complexes.

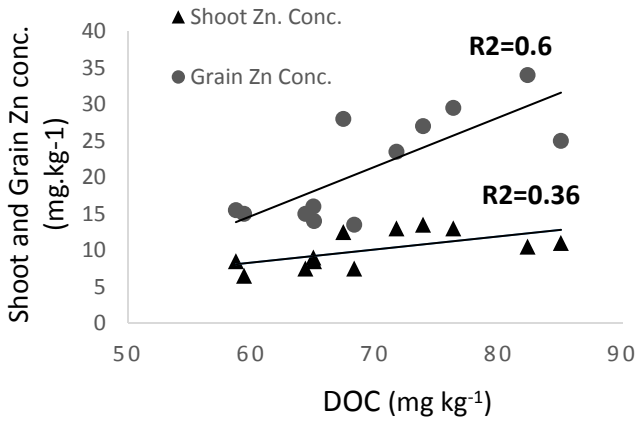

Figure 1- Relationships between soil DOC and Zn in the shoots and grains of Back Cross.

Table 1 – DTPA-extractable Zn in soil, concentrations of shoot and grain Zn, and aboveground biomass production of the wheat cultivars Back Cross and Kavir. Values in the same column with the same letter in a column are not statistically different from each other ($p<0.05$).

Precrop/ressidue treatment	Cultivar	DTPA-Zn (mg kg^{-1})	Shoot Zn conc. (mg kg^{-1})	Grain Zn conc. (mg kg^{-1})	Shoot dry matter (g pot^{-1})	Grain yield (g pot^{-1})
Safflower	Back Cross	0.32 ^{b}	13.0 ^{a}	26.1 ^{a}	16.5 ^{a}	6.9 ^{b}
	Kavir	0.39 ^{a}	8.3 ^{c}	20.8 ^{b}	18.0 ^{a}	9.5 ^{a}
Clover	Back Cross	0.3 ^{b}	11.5 ^{ab}	29.5 ^{a}	16.1 ^{a}	4.8 ^{c}
	Kavir	0.3 ^{b}	8.5 ^{c}	21.1 ^{b}	16.0 ^{a}	9.9 ^{a}
Sorghum	Back Cross	0.29 ^{b}	8.0 ^{c}	19.6 ^{b}	12.9 ^{a}	2.2 ^{de}
	Kavir	0.32 ^{b}	7.3 ^{c}	15.5 ^{c}	13.1 ^{a}	3.2 ^{d}
Sunflower	Back Cross	0.30 ^{b}	10.3 ^{b}	20.8 ^{b}	12.4 ^{a}	2.6 ^{de}
	Kavir	0.36 ^{ab}	7.8 ^{c}	14.1 ^{c}	16.4 ^{a}	3.3 ^{d}
Control	Back Cross	0.23 ^{c}	5.3 ^{d}	14.8 ^{c}	12.7 ^{a}	1.7 ^{e}
	Kavir	0.24 ^{c}	3.3 ^{e}	12.0 ^{c}	16.4 ^{a}	3.6 ^{d}

ACKNOWLEDGEMENTS

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Rational Nitrogen Fertilization Plans of Irrigated Crops in the Nitrates Vulnerable Zones of the Mediterranean Region of Turkey

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INTRODUCTION

Rational nitrogen fertilization plans were suggested for the main crops cultivated in the Mediterranean Region of Turkey. These fertilization plans were developed for the implementation of Nitrates Directive in Turkey to mitigate nitrate pollution. A comprehensive set of factors was considered to formulate a nitrogen balance sheet, amongst which the soil texture, soil organic matter content, and drainage conditions along with irrigation practices. Soil texture classes are needed to determine the appropriate fertilisation dose for the prevailing crops of Mediterranean Region of Turkey. Nitrate concentration in the irrigation water, nitrogen uptake by plants for a targeted yield, nitrogen losses (leaching, emissions), residual nitrogen and the amount of nitrogen mineralisation have been taken into consideration. It can be argued that decreased N fertilization can be applied without significant yield reduction and this is attributed to increased N use efficiency, as a result of N application according to crops' requirements, farming practices, proper application time and splitting of fertilizer in doses.

METHODS

The main components for N fertilization plans are included in the following formula:

$$N_f = N_{req} - [(N_m + N_{in} + N_r) - (N_l + N_d + N_v + N_{runoff})]$$

where: N_f is the recommended N fertilizer; N_{req} is the total N required to produce a crop of a targeted yield; N_m is mineralized N from SOM; N_{in} is the residual plant available inorganic N; N_r is the N input from rainfall; N_l, N_d, and N_v are N losses through leaching, denitrification, and volatilization, respectively, and N_{runoff} is the quantity of N lost by runoff in the sloping areas. N inputs from irrigation water is a factor which affects the recommended nitrogen in irrigated crops and for this reason, nitrogen inputs from irrigation waters were taken into account (m^3/da). Nitrous oxide emissions from commercial fertilizer use were estimated using the following equation: N_2O Emissions = (FC * EC * 44/28), where FC = Fertilizer Consumption; EC = Emission Coefficient.

Denitrification is an important component of N balances in most ecosystems, and in this fertilization plan, an average value 1.68 kg N da^{-1} is suggested (David et al., 2006). De Willigen (2000) developed a regression model to estimate the amount of N leached, which considers the following factors: annual precipitation, clay content, rooting depth, inorganic, residual and mineralized fertilizer, soil organic carbon and N uptake. To calculate the required nitrogen per each crop, a Microsoft Office Excel 2007 spread sheet was compiled which contains a number of factors that can be easily adjusted if data from field experimentation are available. This is necessary because in the future certain coefficients may be substituted by others which will be derived from field experiments.

RESULTS AND DISCUSSION

For each main crop a targeted yield (Table 1) was proposed in cooperation with experts from Soils, Fertilizers and Water Resources Research Institute, Ankara. Another substantial element is the requirements of crops. In the composed fertilization plans a combination of soil organic matter with clay content is proposed for the soil texture classes. Results of the calculations performed regarding the recommended N for the selected crops, are presented in Table2. It must be underlined that the recommended fertilization for each crop varies among provinces, depending on climate conditions,

slope, hydro morphology and soil type. The main N inputs originate from inorganic fertilizers, irrigation water, atmospheric deposition and manure, whilst outputs include crop harvest, nitrate leaching, and denitrification, and the internal transformations of N consist of N mineralization and immobilization. It has been documented that Adana, Antalya and Hatay are the most pollut

ed provinces by nitrates in the Mediterranean region, due to intensive agricultural activities.

Table 1. N requirements of certain irrigated crops of Mediterranean region in Turkey

Crop	Yield (kg /da)	N required (kg N/da*)
Citrus trees	3.500	18
Corn	1.200	24
Cotton	400	20
Melon	3.00	15
Olive trees	1.800	15
Potatoes	3.000	14
Tomatoes	7.000	20

10 da*=1 ha

Table 2. Recommended amount of N fertilization in irrigated crops of Mediterranean region

Crop	N recommended (kg N/da)			
	Soil Class I	Soil Class II	Soil Class III	Soil Class IV
Citrus trees	14.7	13.5	13.1	8.0
Corn	18.9	18.1	18.1	12.8
Cotton	16.2	15.0	14.5	9.7
Melon	12.1	10.6	9.9	4.9
Olive trees	12.3	10.8	10.1	5.3
Potatoes	10.9	9.1	8.0	3.8
Tomatoes	15.1	14.1	13.9	8.4

CONCLUSIONS

The developed spread sheet model can be easily and efficiently used; amendments and alterations can be made accordingly in cases when coefficients from field experimentation become available (N mineralization, N leaching e.t.c.) These coefficients will substitute those derived from solving pedotransfer functions. Modern irrigation systems such as drip and sprinkler irrigation, assist the avoidance of deep percolation, hence can minimise the nitrates pollution of shallow aquifers. It can be argued that N inputs should not be ignored and farmers are suggested to decrease the quantity of N fertilization in most crops. Calculations showed that decreased quantities of N fertilization required for a targeted yield as a result of proper time of application and increasing nitrogen use efficiency by splitting of N fertilizers into several doses.

ACKNOWLEDGEMENTS

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Symplastic and Apoplastic Root Uptake and Translocation to Shoot of Zinc in Wheat and Triticale as Affected by Exogenous Amino Acids

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INTRODUCTION

Plant root exudates significantly affect zinc (Zn) availability for root uptake and further translocation to the aerial parts. An important mechanism is the formation of soluble organo-Zn complexes (Nowack et al., 2006). Little attention has been paid to the effect of amino acids on Zn uptake by plants, although involvement of amino acid chelation in enhancing the rate of root-to-shoot transport of metal ions has been reported. Gramlich et al. (2013) found that Zn uptake from nutrient solution was enhanced in the presence of histidine and provided evidence suggesting that this was at least in part due to the uptake of undissociated Zn−histidine complexes.

A better understanding of how complexes with amino acids in soil solution affect plant Zn uptake may aid in the optimization of plant Zn nutrition. The aim of this study was to investigate in a hydroponic system how application of the amino acids glycine and histidine to the solution would affect apoplastic and symplastic uptake and root-to-shoot translocation of Zn by a triticale and a bread wheat cultivar.

METHODS

Seeds of the bread wheat (*Triticum aestivum*) cultivar Back Cross and the triticale (X. triticosecale) cultivar Elinor were surface sterilized by applying 1% H_2O_2 for 30 min and washed thoroughly with distilled water. After germination the seedlings were transferred into a continuously aerated nutrient solution. When they were 4 weeks old, they were transferred to 600 ml plastic pots and grown for two more days in the basal uptake solutions (without aeration) and then exposed to 10 µM Zn in the form of $ZnSO_4$ in combination with either 50 µM glycine, 50 µM histidine or no amino acid. A treatment without Zn and without amino acids was used for control. The experiments were set up in a completely randomized factorial design. Each treatment was applied in three replicates.

After exposure to the experimental solutions, the seedlings were harvested and separated into root and shoot tissues. The shoots were oven-dried at 72°C for 3 days. The roots were further divided into two aliquots. One was oven-dried without prior washing, while the other was washed with an EDTA solution before oven-drying. All plant samples were analyzed for Zn after microwave digestion by means of AAS. Zinc remaining in EDTA-washed roots was considered as symplastic Zn, whereas Zn desorbed from roots was considered to be apoplastic Zn.

RESULTS

Histidine increased Zn accumulation in the shoots of both plants, whereas glycine had no effect (Figure 1). In wheat this histidine effect was associated with an increased concentration of symplastic root Zn, whereas histidine decreased symplastic Zn in the triticale roots. Glycine decreased symplastic root Zn in both plants. On the other hand, there was no or only very little difference between the effects of the two amino acids on apoplastic root Zn. Both amino acids reduced apoplastic Zn accumulation in the roots of Back Cross wheat, but not in triticale. In triticale, glycine had no effect on apoplastic root Zn, while histidine led to a slight increase. The amino acid effects on total root Zn accumulation were a significant increase in triticale treated with histidine and a decrease in the other cases.

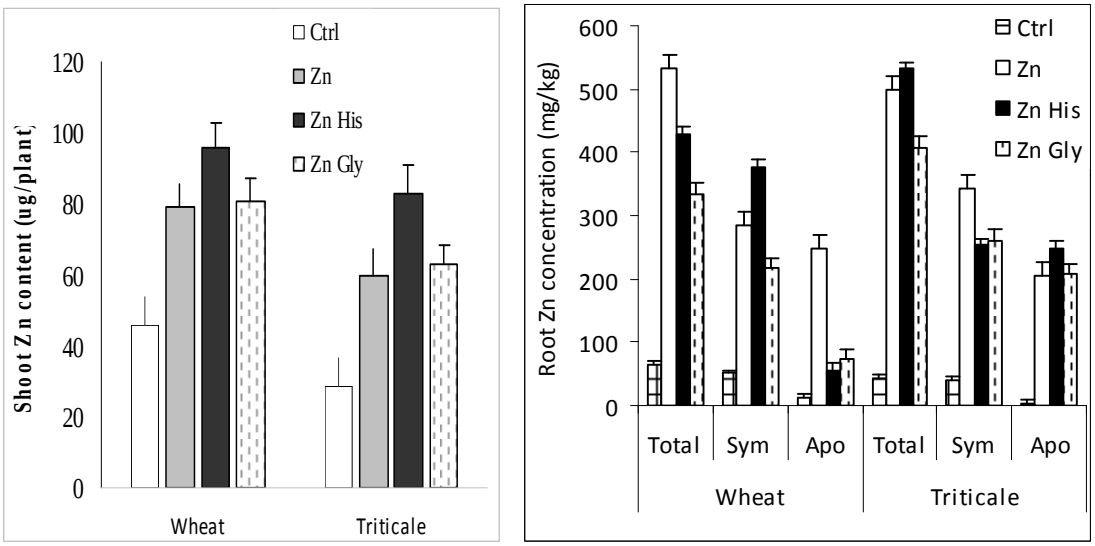

Figure 1: Shoot Zn concentrations (left) and total, apoplastic and symplasmic root Zn concentrations (right) of triticale and wheat in presence or absence of histidine and glycine in hydroponic solution.

DISCUSSION AND CONCLUSION

In contrast to glycine, histidine enhanced Zn accumulation in the shoots of both plant species, whereas the effects of the two amino acids on root Zn did not show a consistent pattern. Although there was no obvious general relationship between the observed amino acid effects on shoot Zn and those on root Zn, the findings show that amino acids with the ability to form strong complexes with Zn such as histidine can have significantly enhance plant Zn uptake and translocation along the symplastic pathway. The variability in the effects to the two amino acids on root Zn suggests that also indirect effects besides the formation of Zn amino-acid complexes played a role.

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Interaction of Selenium in the Soil-Plant System within areas with and without Se Deficiency to Animals

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INTRODUCTION

Romania is situated in a World's area with deficiency selenium values, registered in animals and even people. Thus, Salanţiu, as early as 1970, highlighted the selenium deficiency in calves, lambs, sucking pigs, and young buffalos in large areas from the central-western part of the Country. Serdaru and Giurgiu (2007) established, in the same area, based on thousands of analyses of fodder samples and biological products sampled from animals and people that up to 5% of the analyzed samples belong to the normal content interval. Alike, Serdaru et al. (2003), analyzing similar samples from the south-eastern part of the Country, reach the conclusion that only 6.5% of the analyzed samples belong to the normal content interval, and the difference belongs to deficiency interval. In this zone, in a smaller area in which myodystrophy had occurred in sheep, Lăcătuşu and Ghelase (1992) highlighted deficiency contents in soil and in plants. The objective of the present paper is to establish the selenium level in the soil-plant system from areas in which selenium deficiency occurred in animals and in unaffected areas.

METHODS

The researches had expeditionary character, soil and plants were sampled from seven areas (Table 1). The main sampled plants were pasture plants (areas 1, 3, 4, 7) and straw cereals and maize (1, 2, 3, 5, and 6). The samples were analyzed in the laboratory from the point of view of macro- and microelements contents, through ISO or STAS standardized methods. The selenium contents of soil and plants samples were determined by atomic absorption spectrometry in the boron hydride variant. The mobile soil selenium content was determined in the CH_3COONH_4-EDTA solution at pH 7.0. Total selenium fractions were determined by an original method. The analytical results were statistically computed, using correlations, ANOVA and the Kriging method in a Surfer programme to draw up a tendency map of the selenium distribution in soil.

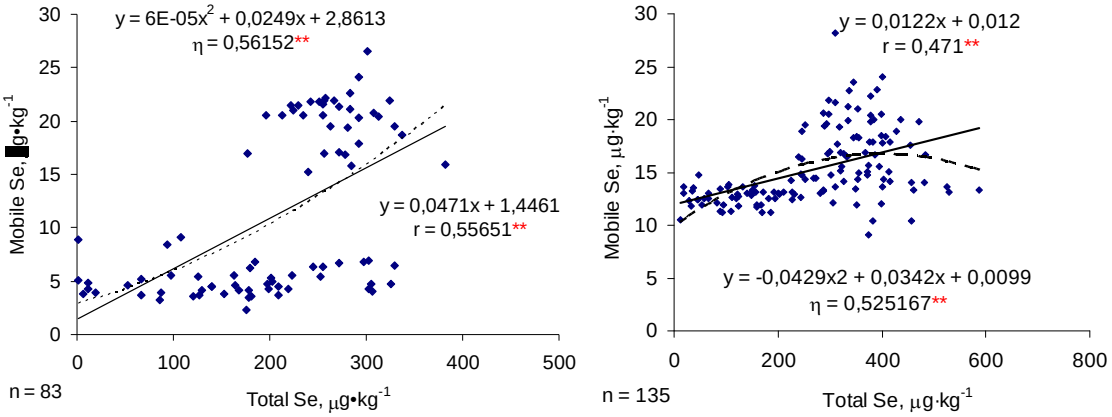
RESULTS AND DISCUSSIONS

Total and mobile selenium abundance in the analyzed soils, represented by the average contents values (Table 1), points out differences up to 623 $\mu g \cdot kg^{-1}$ for total selenium and 20 $\mu g \cdot kg^{-1}$ for mobile selenium. Deficiency values were registered in the areas 1, 2 and 3, where selenium deficiency was registered in animals, especially sheep. In these areas the tendency maps of selenium distribution show contrasting values. Distinctly significant correlations were registered between total and mobile selenium contents in soils (Fig. 1).

The total selenium fractions in a soil from an area with soil selenium deficiency highlighted this element predomination in the fractions hard to mobilize. As a result of the different selenium contents of soil, especially mobile selenium, the plants also accumulated different quantities. Thus, fodder plants from areas 1 and 4 accumulated average selenium quantities, up to 19 $\mu g \cdot kg^{-1}$, four times less than the average 73 $\mu g \cdot kg^{-1}$ value, reported by Kabata-Pendias (2001) for the fodder plants from other parts of the World. Likewise, the straw cereals from area 1 accumulated 22 $\mu g \cdot kg^{-1}$ in the whole green plant and 0.5 $\mu g \cdot kg^{-1}$ in grain. Unlike the area 1, in the areas 3, 5, and 6 the average selenium contents in straw cereals and maize oscillated between 48 and 92 $\mu g \cdot kg^{-1}$. The fact is worth pointing out that between the plants selenium contents and the soil mobile selenium contents distinctly significant relationships have been established with correlation ratios values between 0.623 and 0.792.

Table 1. The average values of the total and mobile selenium contents (μg·kg^{-1}) of the soils from the researched areas

Area	Zone number	Total Se	Mobile Se
Central and Southern Dobrogea	1	143	4
South-Eatern Romanian Plain	2	237	14
Călmăţui and Buzău Rivers Valleys	3	766	18
Făgăraş Depression	4	268	15
Copşa Mică	5	328	22
Livada	6	425	17
Danube Delta, Sireasa and Pardina diked areas	7	600	24



a ***b***

Fig. 1: Correlations between the total and mobile selenium contents in the upper horizon (0-20 cm) of the soils from the south-eastern part of Romania (***a***) and from the Făgăraş Depression (***b***)

CONCLUSIONS

The total soil selenium contents oscillated between 143 and 766 μg·kg^{-1}, and the mobile ones between 4 and 24 μg·kg^{-1}. Between the total and mobile selenium contents direct proportionality relations have been established. Most of the total soil selenium content (81%) is represented by the hardly soluble fractions. The average selenium contents of the pastures in the areas with selenium deficiency in animals were four times lower than the values registered in similar plants from other parts of the World. Between the plants selenium content and soil mobile selenium content correlative relations have been established statistically ensured.

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Calcium Biofortification of Apples – Interaction with Macronutrients

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INTRODUCTION

Calcium is an essential nutrient for humans, having key structural and signalling roles (Dodd et al., 2010). Likewise, low Ca intake in humans has been linked to various diseases (e.g. rickets, osteoporosis, hypertension and colorectal cancer) which can threaten quality of life (Dayod et al., 2010). Biofortification of apples with Ca can be a good method to enhance human intake of Ca and is advocated as an environmentally advantageous strategy (Dayod et al., 2010), yet it can affect the levels of the other macronutrients.This study aims to develop a Ca biofortification method in two of the most economical apple varieties and to assess the implications in macronutrients accumulation.

METHODS

The apple varieties Golden Delicious (G) and Jonagold (JG) were sprayed 10 times before harvest, being applied the following treatments: TC - (control treatment - 0.5% Ca $(NO_3)_2$ + 0.4% $CaCl_2$); TA (0.35% $CaCl_2$ + 1.6% $CaCl_2$) and TB (0.5% $Ca(NO_3)_2$ + 1.6 % $CaCl_2$). Additionally, the apples submitted to treatments TA and TB were post-harvest treated through the immersion of the fruits in a solution of $CaCl_2$ (1.3%) for 5 minutes. All samples were analyzed with the respective epidermis. At different stages of fruit growth, the following nutrients were measured: Ca and Mg (by atomic absorption spectrophotometry), K and Na (by emission photometry) and P (by molecular absorption spectrophotometry). The corresponding values for harvest and postharvest were further confirmed by optical emission spectrometry by inductively coupled plasma. Each value represents the mean of 15 apples with instrumental replicates for each mineral element. Statistical analysis was performed by one-way Anova ($P \leq 0.05$). For mean comparison, a Tukey test was applied, using a 95 % confidence level. Different letters indicate significant differences among treatments.

RESULTS AND DISCUSSION

At harvest, on a fresh weight basis, Ca contents in Golden apples (Table 1) reached 4.5 mg/100g_{fw} in TB, being significantly greater relatively to TA and TC (with 3.3 and 3.1 mg/100g_{fw}, respectively).

Table 1. Calcium contents in Golden (G) and Jonagold (JG) apples submitted to the treatments TA, TB and TC during the growth/maturation, harvest and post-harvest phases.

Assay phases	**Treatments** (mg /100g_{fw})					
	G-TA	G-TB	G-TC	JG-TA	JG-TB	JG-TC
Pulverisation number						
1st	11.8± 0.0	11.8± 0.0	11.8± 0.0	14.2± 0.3	14.2 ± 0.3	14.2 ± 0.3
3nd	6.9±0.1^{a}	6.7± 0.2^{a}	6.5± 0.1^{a}	5.3 ±0.1^{b}	5.7 ± 0.2^{a}	4.7 ± 0.0^{c}
6th	4.0± 0.0^{b}	4.6± 0.1^{a}	3.2 ± 0.1^{c}	3.2 ±0.4^{a}	3.6 ± 0.0^{a}	2.6 ± 0.0^{b}
9th	4.4 ± 0.1^{a}	4.2± 0.1^{a}	3.7± 0.7^{a}	3.2 ±0.1^{a}	2.2 ± 0.0^{b}	2.2 ± 0.1^{b}
Harvest	3.3 ± 0.1^{b}	4.5± 0.1^{a}	3.1± 0.0^{c}	4.3 ±0.1^{a}	3.3 ± 0.0^{b}	3.0 ± 0.1^{c}
Post-Harvest	4.9± 0.1^{b}	6.2± 0.1^{a}		4.0 ±0.0^{b}	4.8 ± 0.1^{a}	

Each value is the mean ± S.D (n=2) of 15 apples. Different letters (a,b,c) indicate significant differences ($p \leq 0.05$) among treatments within each variety.

Post-harvest treatment, further increased Ca levels in TB, but TA also increased (about 50%). Besides, at harvest, Ca contents in the Jonagold apples variety (Table 1) revealed 4.3 mg/100g_{fw} in TA, which was found to be significantly higher than TB and TC (3.3 and 3.0 mg/100g_{fw}), respectively. Moreover, at post-harvest TB displayed a significantly higher content than TA, increasing its amount about 48%.

At harvest and on a dry weight basis, the Golden apples revealed (Table 2) a significantly higher amount of Ca in treatment TB (relatively to TA and TC). The levels of K and P showed similar levels in TA and TB, but significantly higher relatively to TC. Moreover, Mg stood out with a higher content in TC (relatively to TA and TB), whereas Na showed a higher content in TA. At post-harvest, the Golden apples further revealed (Table 2) a significantly higher amount of Ca, relatively to TA. A significantly higher content of P was also found for TB, whereas no significantly variations were found with K. Na and Mg remained significantly higher in TA.

At harvest and on a dry weight basis, Ca in Jonagold apple variety (Table 2) decreased significantly according to the pattern JG-TA>JG-TB>JG-TC, but Na and K did not vary significantly. P showed a higher content in TB, whereas Mg in TC. At post-harvest (Table 2) Ca, Mg and P kept significantly higher in TB, but the opposite occurred with Na and no significant differences were found for K.

Table 2. Macronutrients contents in Golden (G) and Jonagold (JG) apples submitted to the treatments TA, TB and TC at harvest and post-harvest.

Macronutrients	**Treatments** (mg /100g_{dw})					
	G-TA	G-TB	G-TC	JG-TA	JG-TB	JG-TC
			Harvest			
Ca	21.3±0.5^{b}	28.9±0.4^{a}	20±0.7^{b}	25.3±0.4^{a}	19.4±0.2^{b}	17.6±0.7^{c}
K	583±8^{a}	606±10^{a}	497±4.2^{b}	699±195^{a}	578±25^{a}	571±39^{a}
Mg	25.8±0.5^{b}	25.5±0.9^{b}	28.7±0.4^{a}	29.0±0.1^{c}	30.9±0.3^{b}	36.0±0.5^{a}
Na	1130±4^{a}	446±67^{b}	358±5^{b}	520±61^{a}	739±16^{a}	824±323^{a}
P	52.9±0.9^{a}	54.3±0.4^{a}	47±0.4^{b}	53.5±0.1^{c}	61.8±1.0^{a}	57.8±0.7^{b}
			Post-harvest			
Ca	32.0±0.3^{b}	40.2± 0.5^{a}		24.0±0.0^{b}	28.6±0.8^{a}	
K	582±13^{a}	572±18^{a}		558±2^{a}	549±32^{a}	
Mg	25.5±0.0^{a}	24.8±0.2^{b}		26.7±0.1^{b}	31.0±0.4^{a}	
Na	481±12^{a}	315±26^{b}		710±9^{a}	419±35^{b}	
P	53.7±0.7^{b}	59.4±0.6^{a}		45.7±0.0^{b}	56.1±0.6^{a}	

Each value is the mean ± S.D (n=2) of 15 apples. Different letters (a,b,c) indicate significant differences ($p \leq 0.05$) among treatments within each variety and harvest or post-harvest.

CONCLUSIONS

At harvest, TB is the best Ca treatment to achieve a high biofortification in the Golden variety, but in Jonagold TA was found to be more promissing. Moreover, through additional immersion of the fruits in a solution of $CaCl_2$ (1.3%), higher Ca contents can be achieved. Yet, different interactions with macronutrients occur, prevailing heterogeneous accumulation patterns.

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Calcium Biofortification of Apples – Interaction with Micronutrients

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INTRODUCTION

Calcium is an essential nutrient for humans, having key structural and signalling roles (Dodd et al., 2010). Low dietary Ca intake has been linked to several diseases (Dayod et al., 2010). Biofortification of apples with Ca, although being a good method to enhance human intake of Ca (Dayod et al., 2010), can affect the levels of the other micronutrients. This study aims to assess the implications of micronutrients accumulation in two Ca biofortified apple varieties (Golden and Jonagold) having a high economical value.

METHODS

The apple varieties Golden Delicious (G) and Jonagold (JG) were sprayed 10 times before harvest, being applied the following treatments: TC - (control treatment - 0.5% Ca $(NO_3)_2$ + 0.4% $CaCl_2$); TA (0.35% $CaCl_2$ + 1.6% $CaCl_2$) and TB (0.5% $Ca(NO_3)_2$ + 1.6 % $CaCl_2$). Additionally, the apple varieties submitted to treatments TA and TB were post-harvest treated through the immersion of the fruits in a solution of $CaCl_2$ (1.3%) for 5 minutes. All samples were analyzed with the respective epidermis. At different stages of fruit growth, the following nutrients were measured by atomic absorption spectrophotometry: Ca, Fe, Mn, Zn, Cu and B. The corresponding values for harvest and post-harvest were further confirmed by optical emission spectrometry by inductively coupled plasma. Each value represents the mean of 15 apples with instrumental replicates for each mineral element. Statistical analysis was performed by one-way Anova ($P \leq 0.05$). For mean comparison, a Tukey test was applied, using a 95 % confidence level. Different letters indicate significant differences among treatments.

RESULTS AND DISCUSSION

At harvest and post-harvest, the amount of Ca in Golden apples was found to be higher in TB (relatively to TA and TC), with 39% and 50% increases occurring after immersion, in 1.3% $CaCl_2$, of TB and TA, respectively (Fig. 1).

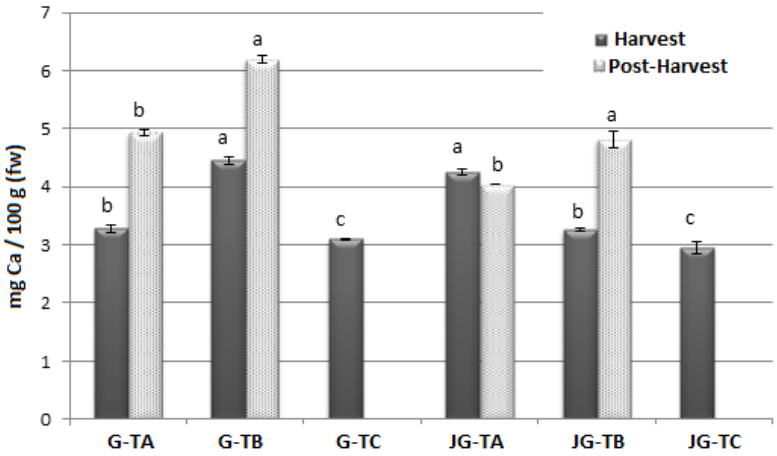

Fig. 1. Ca contents in Golden and Jonagold apples, at harvest and post-harvest, submitted to TA,TB e TC. Each value is the mean ± S.D. (n=2) of 15 apples. Different letters (a,b,c) indicate significant differences ($p \leq 0.05$) among treatments within each variety and harvest or post-harvest.

On the other hand, at harvest, Jonagold apples showed (Fig. 1) a significantly higher amount of Ca in TA (relatively to TB and TC), but at post-harvest a different pattern was found with TB displaying a higher amount of Ca (i.e., after immersion in $CaCl_2$, TA increased 5%, whereas TB augmented 48%).

At harvest, the content of trace elements in Golden apples showed (Table 1) significant differences: Fe displayed higher levels in TA; Mn kept lower in TA; Zn remained lower in TB; Cu had minimum values in TC. Moreover, B did not vary significantly. The content of trace elements Fe, Mn, Cu and B after the post-harvest treatment of Golden apples showed (Table 1) no significant differences between TA and TB, however Zn displayed a statistically higher level in TB.

At harvest, the levels of Cu and Mn in Jonagold apples were found (Table 1) to be higher in TC (relatively to TB and TC), whereas for Fe, B the lowest values were detected in TA. Following the post-harvest Ca treatment, Cu and Fe showed a higher content in TB (relatively to TA), Zn and B did not vary significantly between TA and TB, and Mn prevailed in TA.

Table 1. Micronutrients contents in Golden (G) and Jonagold (JG) apples submitted to the treatments TA, TB and TC at harvest and post-harvest.

Macronutrients	**Treatments** (mg /100g_{dw})					
	G-TA	G-TB	G-TC	JG-TA	JG-TB	JG-TC
			Harvest			
Fe	1.7±0.3^{a}	1.1±0.1^{b}	0.7±0.1^{b}	0.8±0.1^{b}	1.2±0.0^{a}	1.2±0.1^{a}
Mn	0.3±0.0^{c}	0.4±0.0^{b}	0.5±0.0^{a}	0.5±0.0^{b}	0.5±0.0^{b}	0.6±0.0^{a}
Zn	0.3±0.0^{a}	0.2±0.0^{b}	0.3±0.0^{a}	0.3±0.0^{b}	0.3±0.0^{a}	0.3±0.0^{a}
Cu	0.2±0.0^{a}	0.2±0.0^{a}	0.1±0.0^{b}	0.2±0.0^{b}	0.2±0.0^{b}	0.3±0.0^{a}
B	2.8±0.5^{a}	2.0±0.2^{a}	2.0±0.2^{a}	1.7±0.0^{b}	2.0±0.0^{a}	20.2±0.4^{a}
			Post-harvest			
Fe	1.1±0.5^{a}	1.5±0.3^{a}		0.7±0.0^{b}	1.1±0.1^{a}	
Mn	0.4±0.0^{a}	0.5±0.0^{a}		0.6±0.0^{a}	0.5±0.0^{b}	
Zn	0.2±0.0^{b}	0.3±0.0^{a}		0.2±0.0^{a}	0.3±0.0^{a}	
Cu	0.2±0.0^{a}	0.2±0.0^{a}		0.2±0.0^{b}	0.3±0.0^{a}	
B	2.9±0.2^{a}	2.2±0.3^{a}		2.0±0.2^{a}	2.0±0.1^{a}	

Each value is the mean $\pm$ S.D. (n=2) of 15 apples. Different letters (a,b,c) indicate significant differences ($p \leq 0.05$) among treatments within each variety and harvest or post-harvest.

CONCLUSIONS

At harvest, TB is the best Ca treatment for achieve a high biofortification in the Golden variety, but in Jonagold TA was found to be more promising. The translocation and accumulation of trace elements within each variety display different patterns. Besides, after the post-harvest treatment, $CaCl_2$ (1.3%) further affects its contents. Accordingly, if Ca biofortification is triggered in apples, the implications in trace elements accumulation must additionally be considered.

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Possible Protection Abilities of the Plant Cell Wall in Abiotic Stress

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INTRODUCTION

During the last time, an outstanding increased interest can be noticed in research of biopolymers from renewable sources. The future shortage of natural energy sources and replacement of petroleum-based products connect with the solution of worldwide environmental problems. The demands for healthy food, feed, and alternative medicines are the main driving forces for the immense activities in research of polysaccharides, including hemicelluloses (Ebringerová et al. 2005).

The plant biomass, built predominately of cell walls, has been pointed out as the most important source for a broad variety of advanced polymeric materials (Ebringerová et al. 2005) utilizable in various branches of industry and environmentaly harmless technologies. However, many of them have also shown to have biological activity in living systems in a range of physiological processes as growth and development, or protection against the environmental factors, pathogens or abiotic stresses.

Galactoglucomannan oligosaccharides (GGMOs, used in our studies) belong to the group of biologically active oligosaccharides known as regulatory or signaling molecules. These oligosaccharides influence the growth and development of plant roots, aboveground plant parts, vitality, division and differentiation of cells and protoplasts.

They improved also growth parameters of cadmium treated plants. Cadmium is a toxic metal taken up by roots, and transported to shoots, negatively affecting physiological processes.

The aim of this contribution is to give a summary of our actual results on the assumed participation of the plant cell wall and derived oligosaccharides in the protection of the plant, which were obtained during the COST Action FA0905.

MATERIAL AND METHODS

In experiments *Zea mays* – two hybrids - sensitive and tolerant, *Brassica juncea*, *Brassica napus*, *Arabidopsis thaliana*, and *Vigna radiata* were used. Chemical methods for isolation of cell wall components, separation methods, HPLC, analytical methods of polysaccharides identification, fluorescent microscopy, histochemical methods, method for pigment determination, plant growth analysis, AAS method and inductively coupled plasma mass spectroscopy ICP-MS analysis for cadmium determination were utilized.

RESULTS AND DISCUSSION

Plant cell wall

The plant cell wall is an important cell feature that performs numerous essential functions (Keegstra, 2010). As the first barrier it prevents the input of toxic metals into the cell and plant organ, but the defense mechanism is still unknown. In this connection it was determined that cadmium cations cause changes in polysaccharides composition and their quantity in root cell walls of maize. The highest accumulation of cadmium cations in cell wall polysaccharide fractions occurred in alkaline extracted fractions. The cations were probably complexed mainly with glucuronic acid of glucuronoarabinoxylan.

GGMOs and toxic metal stress (cadmium)

GGMOs showed a protective effect on the elongation, growth dynamics, pigment content, fresh and dry mass production, and structure of primary roots under stress induced by Cd^{2+}. GGMOs induced later

development of xylem, lateral roots, and root hairs formation in *Arabidopsis*. Later maturation of the root caused faster growth and uptake of water and solutes. GGMOs and silicon in plants protection against cadmium toxicity showed different ways of suberin lamellae development indicating the existence of diverse protective mechanisms activated by these substances. Differences in root structure under cadmium treatment were species specific. Comparisons were performed in tissues of intact plants between cultivars of *Zea* and *Brassica* species. GGMOs didn't affect the development of suberine lamellae, distribution and accumulation of cadmium in *Arabidopsis*. Therefore it is assumed that the protective effect of GGMOs has a metabolic basis.

CONCLUSIONS

From changes in the cell wall polysaccharides composition under metal stress it is clear, that the cell wall serves not only as a sink for toxic metal accumulation, but is also extensively modified.

GGMOs have shown a protective effect against Cd ions, however, they didn't affect the distribution, neither the accumulation of Cd in plants, therefore a metabolic basis of their protective effect is assumed.

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Uptake of Cd by Contractile Roots Differs from Usual Roots

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INTRODUCTION

Occurrence and function of contractile roots is usually connected and discussed in literature with respect to the process of shrinking of basal parts of these specific roots and pulling the shoots belowground. It is best known in bulbs, protecting these reserve organs against herbivores and harsh environmental impacts. Process of contraction is subject to dimensional changes of cells in contracting basal part of roots. This also requires specific anatomical adaptation of root base with less lignified and suberized tissues in comparison with subapical part of the root body. This basal parts are more active in water uptake than apical-subapical parts which is in contrast to regular root types (North and Nobel 2000). We investigated if the basal parts are also more active in uptake and translocation of cadmium.

METHODS

Plants of the medicinal plant *Tritonia gladiolaris* were cultivated in agar (Ascough et al. 2010). For the experiment the agar was divided horizontally into two parts. In the first variant the upper part contained 50 µmol Cd(NO3)2) and the lower was Cd free, in the second variant 50 µmol Cd(NO3)2) was in the lower part and the upper part was Cd free. Anatomical analysis of root was performed and Cd content was determined by the ICP-MS. The same experimental design: separated compartments of agar with or without 50 µmol Cd(NO3)2) was used also for cultivation of maize seedlings.

RESULTS AND DISCUSSION

The unusual developmental characteristics are accompanied by more intensive uptake of Cd ions by the basal part of contractile roots in comparison with the apical-subapical root part as it was found in experiment with *Tritonia gladiolaris*. The same experimental design: separated horizontal compartments of agar with or without 50 µmol $Cd(NO_3)_2$) resulted in non-contractile roots of model plant (maize) to the opposite uptake and transport characteristics: higher by the apical-subapical root part and lower by the basal root part.

CONCLUSIONS

Specific characteristics of contractile roots may have great impact on uptake of ions, including toxic metals, from the soil surface, which is important for plant nutrition and also for the food safety.

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Performances of Gentle (Phyto)remediation Options at Field Scale in the EU FP7 GREENLAND Network of Trace Element-Contaminated Sites

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INTRODUCTION

Performances of the most promising gentle remediation options (GRO) for trace element-contaminated soils (TECS), i.e. (aided) phytostabilisation, phytoextraction, and *in situ* stabilization/phytoexclusion, are assessed in a European network of 14 large field trials, within the EU FP7 GREENLAND project (http://www.greenland-project.eu/). The GRO efficiency is evaluated regarding various (a)biotic stresses, climatic conditions, pollutant linkages, (phyto)remediation strategies and sustainable land management. Investigated field sites cover a range of contamination scenarios (e.g. agricultural soils contaminated by dust fallout, sludged soils, mine tailings, landfills, dredged sediments, and post-industrial soils).

METHODS

Harmonized datasets are built up on metal(loid) exposure, plant parameters and yields (notably for plant parts converted into feedstock), mineral and biochemical composition of plant parts, ecosystem services, financial return and costs. Soils are sampled to monitor changes in metal(loid) exposure (e.g. labile contaminant pools), transfer to environmental compartments and bioaccessibility, ecotoxicological risks, and soil (multi)functionality and biodiversity. Transfer and bioconcentration factors, shoot metal(loid) removal, contaminant fluxes, and tolerance indices are computed. Dose (exposure) – plant response relationships are modelled.

RESULTS AND DISCUSSION

Data are summarized for various plant covers including poplar and willow short rotation coppices, annual crops of secondary metal accumulators (sunflower and tobacco), and metal-excluders (e.g. perennial grasses, barley and maize cultivars). The long-term efficiency and sustainability of GRO, progresses in remediation objectives (in compliance with national and best procedures), timescale management, maintenance, uncertainty and limitations (including spatial variation of contaminants, water requirements, global changes, etc.), potential flexibility and deployment at other sites are discussed as well as new deployed GRO and cultural practices (e.g. bioaugmentation).

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Influence of Different Non-Standard Fertilizers on Yield and Nutritive Values of Seeds of two Soybean Genotypes

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INTRODUCTION

According to Serbian legislation for fertilizers, so-called "non-standard and special products" include many different classes of compounds and mixtures of substances (phytohormones, amino acids, humic acids, inhibitors of nitrification, plant extracts, homoeopathic solutions, vitamins, etc.). That class of fertilizers mainly has indirect influence on plant nutrient metabolism, but they affect crop growth in strong manner. The impact of some fertilizers from this group on alterations of Mg, Fe, Zn and Mn, as well as phytic acid and β-carotene in grain of two soybean cultivars were tested.

METHODS

The trial included application of recommended doses of different plant extracts (Eko-Fert, Agrostemin Zlatni, Algaren BZn, Zircon), fertilizers with humic substances (Zlatno inje), amino acid fertilizer (Amalgerol Premium), phytohormone fertilizer (Epin Extra, based on 24-epibrassinolide), as well as so-called CO_2 foliar fertilizer (Lithovit Forte – $CaCO_3$ nanoparticles). They were applied at the beginning of flowering (first half of June) on two soybean varieties, Nena (standard variety) and Laura (variety lacking in Kunitz trypsin inhibitor). Experiments were performed in rain-fed conditions on chernozem soil type, at the vicinity of Zemun Polje in following seasons: 2009 (cv. Nena), 2011 (cv. Laura), and 2012 (both cultivars). Seasons 2009 and 2011 were moderate for SE Europe in average temperature and precipitation amount, but 2012 was dry with high temperatures, particularly during flowering and grain filling period. After harvesting, average grain yield was assessed, and contents of different metabolites in soybean grain, such as inorganic phosphorus - P_i, phytate (by method of Dragicevic et al., 2011) and β-carotene (colorimetrically, AACC, 1995), as well as the following elements: Fe, Mn, Zn, Mg (by Inductively Coupled Plasma - Optical Emission Spectrometry) and total P (colorimetrically, by method of Pollman, 1991).

RESULTS AND DISCUSSION

Cultivar Nena was treated with Eko-Fert, Agrostemin Zlatni, Zlatno inje and Amalgerol Premium. In 2009, as year with moderate conditions, the yield varied from 3.1-4.5 t ha^{-1}, with the significantly highest values obtained in Zlatno inje treatment. The highest Mg and Mn content in grain were also achieved in "Zlatno inje" treatment (3% and 12% respectively, compared with control). The highest Fe, Zn and total P content was obtained in the control and plants treated with "Amalgerol Premium" and "Agrostemin Zlatni". The highest phytate and β-carotene content was noticed also in control plants, while the highest P_i content was in soybean treated with "Zlatno inje" fertilizer (30% in relation with control). In 2012, as extreme year in meteorological conditions, grain yield was in the range 2.2-3.3 t ha^{-1}, but also with the highest yield realised with application of "Zlatno inje" fertilizer. Other than grain composition achieved in 2009, in 2012 the significantly higher Mg, Mn, Zn and total P contents were obtained in soybean plants treated with "Zlatno inje", "Agrostemin Zlatni" and "Eko-Fert" fertilizers. The higher Fe, phytate, P_i and β-carotene content were also observable in the control plants, like in 2009. This could refer to relatively high physiological activity of humic substances present in "Zlatno inje", which could increase absorption and/or translocation, mainly of Mg and Mn, into soybean grain, together with providing of its

nutritional value with increased P_i content. And finally, the highest ratios between phytate, analysed elements and β-carotene were mainly present in control plants in both years, indicating that tested fertilizers showed positive impact on possible availability of analysed elements.

Cultivar “Laura” was treated with Agrostemin Zlatni, Algaren BZn, Zircon, Epin Extra and Lithovit Forte. In 2011, as moderate season in meteorological conditions, yield varied between 4.0-5.0 t ha^{-1}, but only in “Zirkon” treatment it was significantly higher (up to12%, compared to control). Mg content in soybean grain was highest also in “Zirkon” (7%, compared to control).The highest Fe and Mn contents were achieved in plants treated with “Algaren BZn” plant extracts (12% and 16% respectively, compared to control). The highest Zn content was recorded in plants treated with “Agrostemin Zlatni” (up to 20% in relation to control). The highest phytate and P_i contents were observable in grain of control plants, but highest β-carotene content was noticed in “Lithovit Forte” treatment. In 2012, the year with high average daily temperatures and low precipitation, grain yield ranged from 1.8-2.8 t ha^{-1}, with the highest value achieved with “Zircon” fertilizer, too, like in 2009. The same fertilizer also showed the highest impact on Mg and Fe accumulation in grain (12% and 20%, respectively, compared to control), while treatment with “Algaren BZn” and “Agrostemin Zlatni” had higher influence on Mn and Zn accumulation respectively (up to 31% and 38%, compared to control). In control, the highest total P and phytate contents were observable, while the highest P_i and β-carotene contents were recorded in grain of plants treated with “Lithovit forte” and “Agrostemin Zlatni”, meaning that these fertilizers could improve availability of examined elements. It was also important to underline that in control plants, ratio between phytate, examined elements and β-carotene had the highest values like in “Nena” cultivar.

CONCLUSIONS

Variations in essential elements and secondary metabolites in soybean grain (important for human and animal nutrition) indicate perspective for application of foliar non-standard fertilizers. Main goal of this agricultural practice is to increase nutritive value of different soybean genotypes irrespective to their genetic background, like variety “laura”; which lacks in kunitz trypsin inhibitor. This is primary related to variable agro-ecological conditions, where grain yield and nutritive value, reflected through better availability of elements, could be jointly increased by spraying with foliar fertilizers.

ACKNOWLEDGEMENTS

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Iron, Zinc, and Phytic Acid Content of Selected Brazilian Soybean Varieties

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INTRODUCTION

Iron (Fe) and zinc (Zn) are some of the most important micronutrients in human nutrition and their deficiency is a well-known health problem worldwide. Biofortification is an important strategy for improving the mineral status of staple foods with respect to micronutrients, namely Fe and Zn. Soybean (*Glycine max*) is a crop that has satisfactory amounts of proteins, carbohydrates, lipids, vitamins, and minerals. As a result, its enrichment with Zn and Fe would be an alternative to combat malnutrition. However, this crop is also rich in phytic acid, an antinutrient that can limit the bioavailability of Fe and Zn. This study evaluated Fe, Zn, and phytic acid contents in soybean grains to identify potential varieties for biofortification purposes.

METHODS

The varieties of soybean grain samples were collected from an experimental field ah the Cooperativa Agroindustrial dos Produtores Rurais do Sudoeste Goiano – COMIGO, located in Rio Verde (GO), Brazil. The experiment was conducted in field plots of 0.5 m x 10 m arranged in randomized block design with three replicates. The plots were previously fertilized with 400 kg ha^{-1} of the commercial fertilizer NPK 2-20-18 applied at the start of growing season and then cultivated with 24 soybean varieties.

For Fe and Zn analyses, the samples were microwave digested according to U.S. Environmental Protection Agency Method 3051A (USEPA, 1998). The Fe and Zn contents in the digested solutions were determined by flame atomic absorption spectrometry. The phytic acid analyses followed Blair *et al*. (2012) with modification. All results are expressed as the means of three replicates. The treatment effects were determined by an ANOVA and Scott-Knott's test to investigate statistically significant differences at $p \leq 0.05$.

RESULTS AND DISCUSSION

The average Fe content in soybean grains was 78 mg kg^{-1} (ranging from 58 to 163 mg kg^{-1}), whereas that of Zn was 40 mg kg^{-1} (varying from 31 to 48 mg kg^{-1}) (Fig.1). Wiersma and Moraghan (2013) found in soybean seeds an average Fe and Zn content of 70 and 34 mg kg^{-1}. For Phytic Acid, the contents values varied between 7.7 and 11.8 g kg^{-1} (Fig.2). There were no significant correlations between the Zn, Fe, and phytic acid contents in the grains. Low contents of phytic acid in soybean grains have the potential to improve the nutritional value of soybean (Yuan *et al*.; 2009).

CONCLUSIONS

Our results suggest that Fe, Zn, and phytic acid contents in soybean varieties are influenced by genetic variability. Also, such results reveal that antinutricional factors, like phytic acid, need to be taken into considerations for biofortification of soybeans with iron and zinc. Yet, further studies are needed in order to address the role of phytic acid upon the bioaccessible contents of Zn and Fe in the studied varieties.

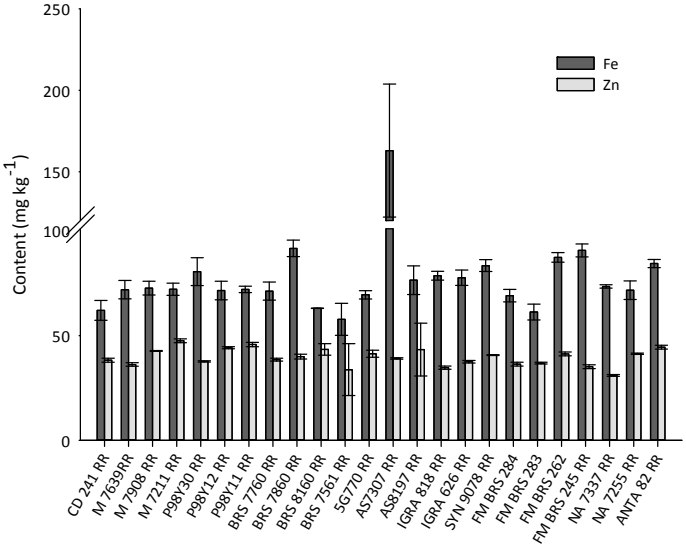

Fig. 1: Grain Fe and Zn contents in soybean varieties

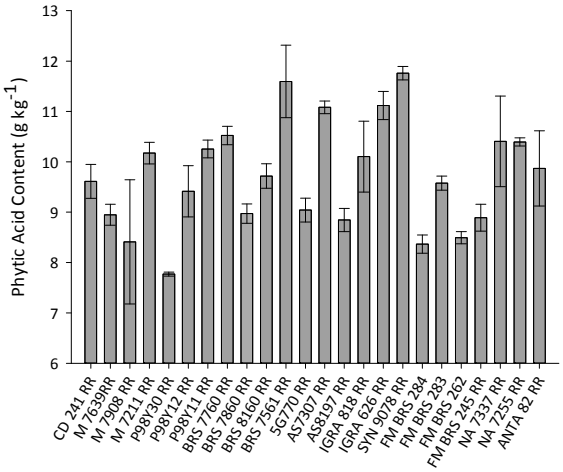

Fig. 2: Grain phytic acid content in soybean varieties.

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Aided Phytostabilisation of a Cr, Mo and Ni-contaminated Technosol

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INTRODUCTION

Aided phytostabilisation uses excluder plants, their associated microorganisms, and soil conditioners to minimize pollutant linkages, notably metal(loid) transfer through natural agencies in the environmental compartments and into the food chain. It aims to reduce adverse effects of contaminants on living organisms and to restore soil ecosystem services. A large-scale aided phytostabilisation program named PHYSAFIMM is developed on metal(loid)-contaminated tailings (30 ha) of a steel smelter located at Rive de Gier, France [1]. This phytoremediation option should restore a vegetation cover, reduce labile soil Cr, Ni and Mo concentrations, and decrease exposure pathways. This study aimed at assessing in controlled conditions the efficiency of Ni/Cd-tolerant plants in mixture and soil amendments to perform aided phytostabilisation on a metal (Cr, Ni, and Mo)-contaminated technosol, while avoiding to increase herbivore exposure.

METHODS

Experimental design

Plots (50 m^2) were implemented in triplicates on the Rive de Gier tailings, with three soil treatments: untreated soil (UNT, pH 11.8), *Ramial chipped wood* (RCW, 160 t/ha, pH 9.4) and composted sewage sludge (CSS, 500 m^3/ha, pH 9.3). Topsoils (25 cm) were collected in these plots and an uncontaminated kitchen garden (CTRL, pH 5.6), Gradignan, France, and placed in pots. Potted Unt soil was also amended with two treatments, i.e. vermiculite (5%) with compost (5%) made of pine bark and poultry manure (VOM), and iron grit (1%) with compost (5%) (OMZ). All potted soils were placed under controlled conditions in a greenhouse. Seeds of a mixed stand including Ni/Cd tolerant populations of *Festuca pratensis* Huds., *Holcus lanatus* L., and *Plantago lanceolata* L. Plants were weekly watered at the field capacity during the experiment.

Soil pore water and shoot ionome

Soil pore water was collected in all pots using 'Rhizon' samplers, and pH and electrical conductivity were measured. After a 15-week growth period, shoots were harvested. Shoot ionome (after NO_3/H_2O_2 wet-digestion) and soil pore water were analyzed by ICP-MS (Thermo X series 200).

RESULTS AND DISCUSSION

The shoot DW yield of *F. pratensis* for the CSS soil was 2 fold higher than that for the CTRL soil: it reached 375 ± 71 g m^{-2} (CSS) compared to 162 ± 5 g m^{-2} (CTRL, Fig. 1). For the UNT, RCW, VOM and OMZ soils, it varied between 12 and 39 g m^{-2}, which was 4 - 10 time lower than for the CTRL soil. After 15 weeks, *F. pratensis* was growing on all soils, whereas *H. lanatus* developed only on the CTRL, UNT and CSS soils and *P. lanceolata* on the CTRL soil. This last one had a recovery rate lower than 5%. Shoot Cr, Ni and Mo concentrations of *F. pratensis* significantly decreased for the CSS treatment compared to the Unt one, i.e. from 31 to 0.8 mg Cr Kg^{-1} , 16 to 1.1 mg Ni Kg^{-1} and 308 to 108 mg Mo Kg^{-1} (Fig. 2). The RCW, VOM and OMZ treatments reduced between 3 and 7 time the shoot Cr concentration of *F. pratensis* (in mg Cr Kg^{-1}), i.e. respectively from 31 to 4, 10 and 6. However, these treatments did not change significantly its shoot Mo and Ni concentrations, which remained between 186 and 376 mg Mo Kg^{-1} and 8 and 16 mg Ni Kg^{-1}.

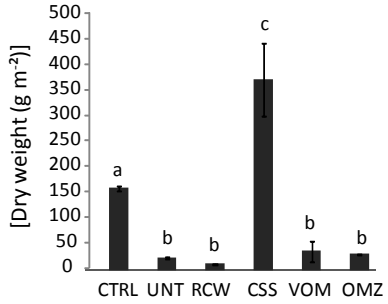

Fig. 1: Shoot dry weight of *Festuca pratensis* (in g m^{-2}) after a 15-week growth period. Mean values per treatment (n=3). Values with different letter differ significantly by one way ANOVA (p-value <0.05)

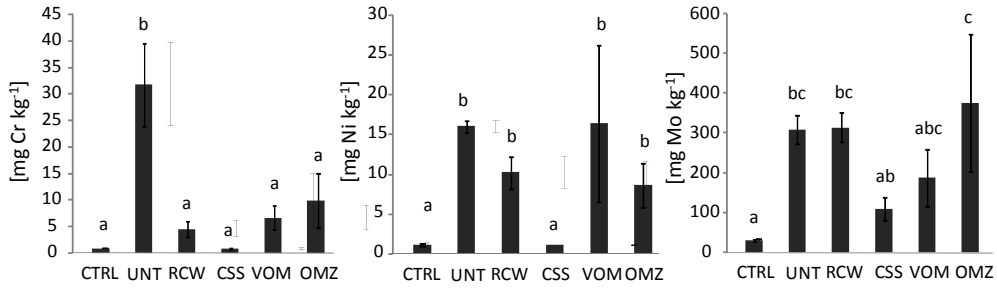

Fig. 2: Shoot ionome of *Festuca pratensis* (mg Kg^{-1}) after a 15-week growing period. Mean values per treatment (n=3). Values with different letter differ significantly by one way ANOVA (p-value <0.05)

The CSS treatment best performed to both increase shoot DW yield of *F. pratensis* and reduce its shoot Cr, Ni and Mo concentration, suggesting a dilution effect. Compared to the Unt soil, soil pore water in the CSS soil indicated decreases in soil pH from 10.2 to 8.7 and Cr exposure and increases in K, Mg, P, and Ni exposures, and soluble organic matter (SOM), which likely improved *F. pratensis* growth. Higher Ni exposure in line with SOM mineralization and higher Mg nutrition may stimulate shoot production of this Ni/Cd-tolerant *F. pratensis* population on the CSS soil compared to the CTRL soil [2]. Other soil treatments did not improve shoot yield of *F. pratensis* and such high shoot Ni, Cr and Mo concentrations may potentially affect herbivores.

CONCLUSIONS

Cultivation of a Ni/Cd-tolerant population of *F. pratensis* on this contaminated technosol amended with CSS is a succesful combination to restore a dense vegetation cover. However the shoot Mo concentration of *F. pratensis* remains elevated. Direct evidences of an aided phytostabilisation of metals or only their in situ stabilization/phytoexclusion need further long-term assessment in plots.

ACKNOWLEDGEMENTS

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On Spatially Resolved Molecular Composition of Buckwheat Grains

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INTRODUCTION

It remains a great challenge to reliably and routinely image spatial distribution of molecular constituents, including both primary and secondary metabolites in plant material without modifying the chemical bonds. Synchrotron Radiation Fourier Transform InfraRed (SR-FTIR) micro-spectroscopy provides information on the sample molecular chemistry with a few micron spatial resolution (diffraction limited) while being non-destructive (Schulz & Baranska 2007). Here we present an approach aiming to determine bulk and tissue molecular make up in buckwheat grain. We studied two buckwheat species usually grown as crop, namely common buckwheat (*Fagopyrum esculentum* Moench) and tartary buckwheat (*Fagopyrum tataricum* Gaertn.). Gluten-free buckwheat grain is of high nutritional quality with a well-balanced amino-acid and favourable lipid compositions and its richness in dietary fibre (Alvarez-Jubete et al., 2009). Tartary buckwheat grain contains lower amounts of total proteins, fats and dietary fibre, but higher amounts of starch and vitamin B than common buckwheat grain (Bonafaccia et al., 2003). However, destructive milling of the grains revealed that the two species differ in molecular composition of specific tissues, bran (comprising cotyledons and aleurone) and flour (endosperm only).

METHODS

Sample Preparation

The grain of a Luxemburg variety of tartary buckwheat (*Fagopyrum tataricum* Gaertn.) and Slovenian common buckwheat (*Fagopyrum esculentum* Moench) cv. Darja were used. For the bulk analyses grains were homogenised with liquid nitrogen using a pestle and a mortar and freeze-dried. For microscopy analyses, grain was soaked for 4 h in Milli-Q water, frozen in liquid nitrogen, cryo-sectioned to 10-µm sections at -25 °C and freeze-dried (details in Pongrac et al., 2013).

Bulk and Tissue-Specific Infrared Analysis of the Molecular Composition

The determination of the main molecular groups present in the entire grain was firstly assessed via whole powdered grain chemical analysis in ATR mode as described in Nečemer et al. (2013). In addition tissue (husk, cotyledon and endosperm) specific spectra were collected from cryo-sectioned grain and data processed as described in Regvar et al. (2013). All the spectra were analysed by application of the OPUS NT 6.5 software package (Bruker). The recorded spectra were corrected due to atmospheric changes, truncated to a range of 3800-800 cm^{-1}, baseline corrected, smoothed, and normalised to permit an accurate comparison of the different spectral domain and their intensity.

RESULTS AND DISCUSSION

The dominant absorption bands of lipids (3,000-2,800 cm^{-1}), proteins (1,750-1,480 cm^{-1}) and carbohydrates (1,180-900 cm^{-1}) and the differences in the intensities and precise peak positions in the two studied species are shown in Figure 1. Spectra of different tissues confirm previously detected differences between the two species.

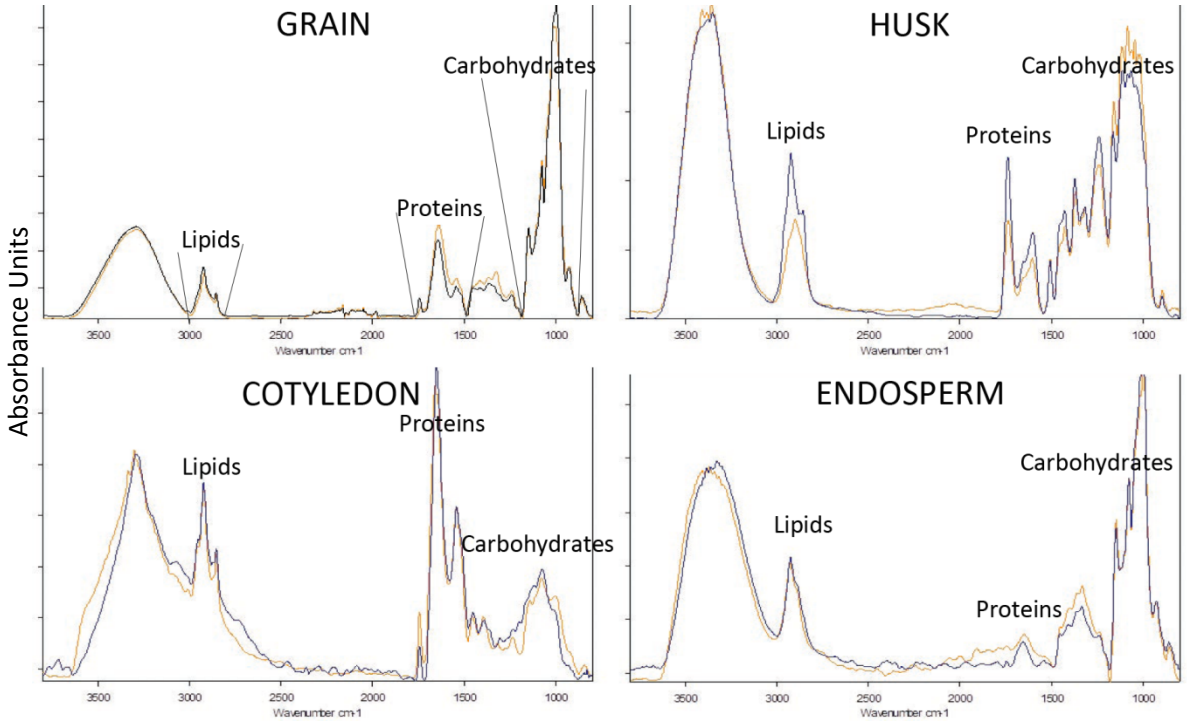

Fig. 1: Comparison of the infrared spectra of tartary (dark line) and common buckwheat (light line). Spectra for whole powdered grain were obtained in ATR mode, while for husk, cotyledon, and endosperm at tissue level the spectra were recorded in transmission with SR-FTIR.

CONCLUSIONS

Higher overall protein and lower carbohydrate amounts in common buckwheat compared to tartary buckwheat grain was attributed to the higher protein and lower carbohydrate amounts found in endosperm, considering the higher relative volume of the endosperm (43 %). Prominent differences were observed in lipid, protein and carbohydrate compositions of husk, which however did not translate directly to overall grain composition due to smaller contribution of this tissue (29 %) to the whole grain. Further detailed analysis will be presented and the differences commented.

ACKNOWLEDGEMENTS

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Zn and Fe Biofortification in *Triticum aestivum* L. – Potential of Photoassimilation

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INTRODUCTION

Metal availability determines Zn and Fe grain accumulation (biofortification) if the threshold of toxicity is not reached, whereas photoassimilates mobilization determines grain filling and, consequently, crop production (field sowing). This work aims at assessing the potential of photoassimilate production of biofortified bread wheat (*Triticum aestivum* L.) genotypes with high potential for field production, using the photosynthetic performance as an evaluation test.

METHODS

Parental (F), F1 and F2, 1st and 2nd generation respectively grains of bread wheat cv. Roxo were sown in a walk-in growth chamber, under environmental controlled conditions (80% RH; 24/20°C day/night temperatures; PPFD of *ca*. 700 µmol m^{-2} s^{-1}, 12 h photoperiod). F (s), F1 (s) and F2 (s) were irrigated with a standard solution (s) until harvest. F1 (5s, 7s, 10, 13s) and F2 (5s, 7s, 10, 13s) plants were irrigated with a standard solution during 1 month after germination, and thereafter with 5, 7, 10 and 13 fold nutrients concentrations until harvest. Scanning electron microscopy (SEM) was performed with a Jeol 330 equipment. Leaf net photosynthetic rate (P_n) and stomatal conductance to water vapour (g_s), were measured with a CO_2/H_2O open system portable IRGA (CIRAS I, PP Systems, UK) with external CO_2 set to 370 µL of CO_2 L^{-1} and PPFD of *ca*. 400 µmol m^{-2} s^{-1}. Chl *a* fluorescence parameters were determined with a PAM 2000 system (H. Walz, Effeltrich, Germany). Photosynthetic electron transport rates were determined at 23-25°C, using a Clark-type oxygen electrode (LW2, Hansatech, Kings Lynn, UK).

RESULTS AND DISCUSSION

Although under the defined growth conditions the contents of Zn and Fe sharply increased in the seeds of F2 (s, 5s, 7s, 10s and 13s) (data not shown), SEM showed that biofortified wheat grains of F2 (7s, 10s, 13s) were shrunken and with low quality and unsuitable for field production Fig. 1).

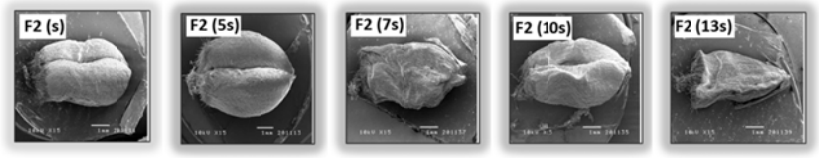

Fig. 1. SEM of F2 bread wheat grains

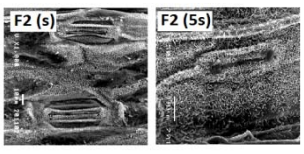

Fig. 2. SEM of F2 stomata.

In this context, to assess the kinetic potential of photosynthate mobilization to the grain, *in vivo* photosynthetic parameters were monitored during growth in F2 (s and 5s), being found that stomata dimensions did not change significantly among treatments (Fig. 2). On the other hand, parameters that reflect the photosynthetic performance, as P_n, F_v/F_m, q_p and $F_{v'}/F_{m'}$ increased in F2 treatments in relation to Fs, particularly at 73 and 91 DAG (Table 1). As g_s decreased the WUE would increase. Moreover,

closer to the life cycle ending, chl *a* (and total chl), and the activities of both photosystems (PSII and PSI), with and without the oxygen evolving complex (OEC), increased in F2 plants in comparison with F(s) (Table 1), further supporting that a high photosynthetic performance was maintained for a longer period.

Table 1. Kinetics patterns of leaves gas exchanges, fluorescence parameters and photosystems activities of F2 (s, 5s). Values are the mean+SE (n=4-6).

	F (s)			F2 (s)			F2 (5s)		
	Days after germination (DAG)								
	55	**73**	**91**	**55**	**73**	**91**	**55**	**73**	**91**
P_n (mmol CO_2 m^{-2} s^{-1})	**10.50**	**11.70**	**7.10**	**16.50**	**18.03**	**16.44**	**15.15**	**16.80**	**16.45**
± S.E.	0.87	0.44	1.39	0.30	0.55	0.35	0.24	0.25	1.37
g_s (mmol H_2O m^{-2} s^{-1})	**892**	**1312**	**2210**	**780**	**899**	**902**	**680**	**708**	**897**
± S.E.	201	209	810	162	120	141	88	125	174
F_o	**0.075**	**0.123**	**0.156**	**0.082**	**0.122**	**0.119**	**0.089**	**0.118**	**0.111**
± S.E.	0.002	0.005	0.006	0.005	0.004	0.004	0.002	0.002	0.003
F_m	**0.432**	**0.608**	**0.534**	**0.516**	**0.734**	**0.694**	**0.533**	**0.729**	**0.693**
± S.E.	0.019	0.027	0.048	0.007	0.032	0.023	0.016	0.028	0.020
F_v/F_m	**0.824**	**0.797**	**0.688**	**0.831**	**0.834**	**0.823**	**0.833**	**0.838**	**0.840**
± S.E.	0.004	0.010	0.041	0.002	0.004	0.009	0.003	0.002	0.001
q_p	**0.783**	**0.824**	**0.773**	**0.889**	**0.890**	**0.845**	**0.906**	**0.905**	**0.891**
± S.E.	0.086	0.029	0.030	0.012	0.020	0.023	0.011	0.005	0.014
q_N	**0.384**	**0.444**	**0.370**	**0.450**	**0.486**	**0.450**	**0.474**	**0.488**	**0.458**
± S.E.	0.066	0.048	0.067	0.020	0.034	0.012	0.031	0.040	0.042
NPQ	**0.409**	**0.550**	**0.327**	**0.513**	**0.634**	**0.585**	**0.630**	**0.675**	**0.609**
± S.E.	0.113	0.128	0.084	0.054	0.087	0.047	0.073	0.120	0.089
$F_{v'}/F_{m'}$	**0.696**	**0.674**	**0.576**	**0.697**	**0.691**	**0.722**	**0.709**	**0.707**	**0.723**
± S.E.	0.024	0.011	0.071	0.008	0.005	0.008	0.010	0.006	0.012
Chl *a* (mg g^{-1}_{fw})	**0.91**	**0.99**	**1.03**	**0.97**	**1.13**	**1.10**	**0.99**	**1.12**	**1.09**
±S.E.	0.10	0.08	0.11	0.07	0.08	0.11	0.09	0.12	0.08
Chl *b* (mg g^{-1}_{fw})	**0.38**	**0.46**	**0.51**	**0.42**	**0.50**	**0.49**	**0.45**	**0.49**	**0.43**
±S.E.	0.03	0.04	0.05	0.05	0.06	0.03	0.03	0.05	0.05
PSII+PSI (mmol O_2 g^{-1}_{fw} h^{-1})	**220**	**240**	**162**	**232**	**240**	**235**	**235**	**245**	**231**
±S.E.	15	18	15	21	19	21	20	19	17
PSII+OEC (mmol O_2 g^{-1}_{fw} h^{-1})	**650**	**703**	**490**	**673**	**697**	**499**	**669**	**705**	**502**
±S.E.	51	65	32	45	51	53	61	44	32
PSII-OEC (mmol O_2 g^{-1}_{fw} h^{-1})	**600**	**640**	**450**	**585**	**667**	**589**	**602**	**624**	**607**
±S.E.	37	51	37	61	57	32	44	56	63
PSI (mmol O_2 g^{-1}_{fw} h^{-1})	**999**	**1121**	**738**	**1068**	**1132**	**895**	**1059**	**1145**	**890**
±S.E.	72	89	65	77	89	66	88	91	57

CONCLUSIONS

In the biofortified plants of F2 (s and 5s), the photosynthetic apparatus displayed a higher level of activity and for a longer time favouring the maintenance of photosynthete production.

Selenium is Accumulated in Chickpea Plants and Modulates Enzymatic and Morphologic Parameters in Greenhouse and Climate Chamber Exposure

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INTRODUCTION

Selenium is essential for the functioning of glutathione peroxidase (GPOX) and thioredoxin reductase (TrxR) in humans, and it is a micronutrient with multiple essential roles in the plant´s antioxidative metabolism. Its concentration in human serum is between 100 and 160 µg/l, and Se deficiency may lead to interference of some organ and tissue functions. The recommended daily supply in Germany, Austria and Switzerland is 30 to 70 µg Se per day. However it is questionable whether Se will be taken up by plants in significant amounts without leading to productivity problems. Chickpea (*Cicer arietinum*) was used as a plant model for fortification due to its use in many Mediterranean diets.

A supplementation of Se in plant based food could be an option to avoid significant Se deficiencies. Therefore several Se concentrations were tested for their effects on the plants and for their availability in the seeds.

MATERIAL AND METHODS

We exposed Chickpea (*Cicer arietinum*), a legume that is basis of food and feed in many countries to elevated Se concentrations and investigated the accumulation of this element in the plant´s tissues. Exposure experiments were carried in semi-hydroponic conditions in a greenhouse and in climate chambers. The daylight/night cycle was set to 16/8 hrs. Temperature control in the climate chambers was set to 23/18 °C during days and nights, respectively. Plants were supplied with $NaSe_2$ solved in tap water in concentrations in the range of 10 to 100 µM for 14 days. After plant harvest and biometrical investigations of shoot and root growth, Fwt/Dwt determination and seed characterization, protein extracts were obtained from root and leaf tissue following published methods (Lyubenova et al. 2009). Selenium contents in the plants was determined from dried samples by ICP-OES. Plant primary production related parameters, photosynthetic performance and water use efficiency were measured according to standard methods with a handheld device (Rühr et al. 2009).

RESULTS

Our study shows that Se is taken up in higher range in chickpea's roots compared to the shoots. Still it can be expected that Se is also accumulated in significant amounts in the seeds, so that is available for human food and animal feed. Exposure with Se between 25 µM and 100 µM is far above the recommended concentrations for agricultural treatment. Hence, these concentrations exceed the plant´s compensatory capabilities and inhibited the plant´s development. The plant´s water use efficiency (WUE) increased with increasing Se supplementation. This was due to stomatal closure, hence plants with higher Se contents obviously had less water available. As a consequence, shoot growth was stunted, and fresh & dry weight was reduced by 30 % after the growing season.

Besides, the activities of several antioxidative enzymes were influenced: We found up-regulation of specific glutathione cycle enzymes, and at the same time decreases in the ascorbate cycle related enzymes. It is evident that elevated Se uptake causes significant stress in chickpea plants but that detoxification in chickpea happens without the participation of the Halliwell-Asade-cycle.

CONCLUSIONS

Selenium fortification in chickpea is possible, and exposure of the plant at very high concentrations is feasible, before stress symptoms appear. Seeds will contain Se concentrations in favorable ranges (mg/kg dwt) for food biofortification. At elevated Se concentrations in the fertilizer or irrigation water, the plant´s primary production as well as its seed production changes. It will be required to set reasonable limits for Se supplementation in the field and to test different chickpea varieties for their accumulation capacity and productivity in the field.

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Efficiency of Biofortification of Lettuce and Carrot with Iodine and Selenium Depending on the Fertilization with various Compounds of these Elements

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INTRODUCTION

Iodine and selenium play important functions in human and animal organisms. A lot of people around the world suffer from its deficit in the daily diet. These elements are not mineral nutrients for plant thus it seems important to develop effective agrotechnical methods of plant biofortification with iodine and selenium [White and Broadley 2009]. The aim of the study was to determine the possibility of performing double biofortification with iodine and selenium of butterhead lettuce and carrot cultivated in field.

METHODS

Studies were conducted in 2012-2013. Both lettuce and carrot were cultivated on a heavy soil.. The study included soil fertilization (of lettuce and carrot plants) with iodine and selenium in the following combinations: 1./ Control, 2./ KI, 3./ KIO_3, 4./ Na_2SeO_4, 5./ Na_2SeO_3, 6./ KI+Na_2SeO_4, 7./ KIO_3+Na_2SeO_4, 8./ KI+Na_2SeO_3, 9./ KIO_3+Na_2SeO_3. Iodine and selenium were applied twice: before sowing and as a top-dressing in a total dose of 5 kg I·ha^{-1} and 1 kg Se·ha^{-1} (presowing and top-dressing as 2.5 kg I·ha^{-1}+0.5 kg Se·ha^{-1}), respectively. Selenium and iodine content in plants samples was determined with the use of high dispersion spectrometer ICP-OES Prodigy Teledyne Leeman Labs. The level of selenium was analyzed after sample mineralization in HNO_3, while iodine – by cold vapor generation technique after sample mineralization in HNO_3 and $HClO_4$.

RESULTS AND DISCUSSION

Tested combinationd with iodine and selenium fertilization had no significant influence on the yield of carrot leaves and storage roots (Table 1). In the case of lettuce, application of Na_2SeO_4 alone or simultaneously with KI or KIO_3 exhibited strong negative influence on yield. This observation suggests that carrot can be described as more tolerant to relatively high doses of SeO_4^{2-} than lettuce. The lower level of iodine and selenium accumulation during carrot and lettuce cultivation was noted after the application of KIO_3 than KI as well as Na_2SeO_3 than Na_2SeO_4. Therefore, relations described in other works have been confirmed.

Iodine accumulation vs selenium application. In the case of carrot, selenium fertilization together with KI lowered the level of iodine uptake/accumulation in leaves and storage roots when compared to the application of KI alone – stronger antagonistic effect was exhibited by SeO_3^{2-} niż SeO_4^{2-}. When applying KIO_3, both forms of selenium comparably decreased iodine level in carrot plants. In lettuce cultivation no such relation were, however, observed. It is worth to underline that for simultaneous application of KIO_3+Na_2SeO_3 a higher content of iodine in lettuce was observed when compared to combination treated with KIO_3 or KIO_3+Na_2SeO_4.

Selenium accumulation *versus* iodine application. In lettuce cultivation, simultaneous application of KI+Na_2SeO_4 or KIO_3+Na_2SeO_4 comparably decreased selenium content in plants when comapred the

application of Na_2SeO_4 alone. No such relations (differences statistically insignificant) were noted for the introduction of SeO_3^{2-} alone or together with KI and KIO_3. In carrot cultivation, however, simultaneous application of KI+Na_2SeO_4 and KIO_3+Na_2SeO_4 (when compared to fertilization with Na_2SeO_4 alone) contributed to a significant increase in selenium accumulation in leaves. Still, it was related to a decrease of Se level in storage roots. With the application of KI+Na_2SeO_3 as well as KIO_3+Na_2SeO_3 a tendency of decreasing the level of selenium in carrot leaves and roots was noted in comparison to the combination with the treatment with Na_2SeO_3 alone. In the studies described in this abstract the assessment of iodine absorption by rats fed with lettuce or carrot biofortified with iodine was also evaluated and the results will be presented in further publications.

Table 1. Yield and content of iodine and selenium in lettuce and carrot plants – means from 2012-2013

Treatments	Lettuce			Carrot leaves			Carrot storage roots		
	Yield	(mg kg^{-1} d.w.)		Yield	(mg kg^{-1} d.w.)		Yield	(mg kg^{-1} d.w.)	
	(t ha^{-1})	I	Se	(t ha^{-1})	I	Se	(t ha^{-1})	I	Se
Control	43.2 b	1.4 a	4.4 ab	22.6 a	1.2 a	2.4 a	101.3 a	2.5 a	0.6 a
KI	46.1 b	4.6 d	1.9 a	21.7 a	7.7 g	1.7 a	97.2 a	15.9 e	1.5 b
KIO_3	45.3 b	3.3 b	3.2 a	24.2 a	5.6 ef	3.7 a	98.5 a	9.6 cd	2.0 b
Na_2SeO_4	26.6 a	1.4 a	128.6 e	24.6 a	2.9 b	55.2 d	106.6 a	2.8 a	25.0 g
Na_2SeO_3	41.9 b	1.6 a	19.7 c	21.1 a	3.5 bc	11.6 c	100.9 a	3.2 a	6.7 e
KI+Na_2SeO_4	28.9 a	4.2 cd	91.0 d	22.0 a	6.2 f	91.3 f	89.2 a	10.7 d	21.4 f
KIO_3+Na_2SeO_4	26.9 a	3.6 bc	97.6 d	22.1 a	4.1 cd	86.5 e	93.1 a	6.6 b	24.6 g
KI+Na_2SeO_3	41.0 b	4.6 d	18.2 bc	20.3 a	5.8 f	10.2 c	94.9 a	9.0 c	3.8 c
KIO_3+Na_2SeO_3	45.2 b	4.5 d	13.1 abc	22.1 a	4.6 de	7.5 b	93.4 a	6.0 b	5.0 d

CONCLUSIONS

Lettuce was more sensitive to applied soil fertilization with SeO_4^{2-} than carrot – this chemical form of selenium was strongly toxic for lettuce plants. Separate mechanisms of iodine uptake/accumulation as affected by selenium fertilization as well as of Se uptake/accumulation *versus* I application was found for lettuce and carrot plants. Previous information suggesting more effective uptake of Na_2SeO_4 over Na_2SeO_3 as well as of KI over KIO_3 have been confirmed.

ACKNOWLEDGEMENTS

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Expression of *AhHMA4p1::AhHMA4* Improves Fruit Yield in the Presence of Cadmium

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INTRODUCTION

Heavy metals which are micronutrients such as Zn, Fe, Ni are essential for plants to grow, however in excess they are toxic. Other heavy metals e.g. Pb, Cd, non-essential for plant development, when present in soil can become highly toxic. Therefore, plants have developed a number of mechanisms to maintain homeostasis of metals to keep under control their uptake and distribution. Proteins involved in the regulation of metal metabolism (transport and chelating processes) are not highly specific for their substrates. Since they bind several metals with different affinity, the same protein is involved in the regulation of homeostasis of several metals. Contribution of the same proteins to the regulation of the metabolisms of different metals was called cross-homeostasis. Genes involved in these proces are frequently used to engineer metal accumulation and distribution pattern. In this study, tomato plants expressing with *AhHMA4p1::AhHMA4* from *Arabidopsis halleri* (which encodes the Zn export protein involved in xylem loading of Zn) (Barabasz et. al 2012) were used to test the usefulness of such transformants in biofortification. Plants were grown in the hydroponic culture (known ion concentration) and on soil (conditions similar to the natural) with and without adding Cd. The following parameters were evaluated: (i) growth and development of plants, (ii) concentration of Zn, Fe, Cd in roots, leaves, fruits and seeds, (iii) expression of selected genes involved in Zn, Fe and Cd homeostasis in roots and leaves.

METHODS

The experiments were conducted on tomato plants (*Lycopersicon esculentum L.*) v. Money-Maker, wild-type and transformed with the *AhHMA4* gene from *A. halleri* under its own promoter (Barabasz et. al 2012). In this study the following growth conditions were used:

(i) **hydroponic culture** (23 days –until development of young seedlings)	• 1/2 Knop's medium	-optimal ions concentration
	•1/10 Knop's medium	-low ions concentration
(ii) **soil** (plants were grown until fruit ripen - 127 days)		

To test plant's response to cadmium, $CdCl_2$ was added to the medium to the following final concentrations: (i) 1 µM in the liquid medium, (ii) 10 mg/kg d.w. of the soil

The examined parameters were as follow: (i) growth and development of plants (at the vegetative and generative phase), (ii) the concentration of Zn, Fe, Cd in roots, leaves, fruits and seeds (plants part were dried then the dry biomass was determined, next samples were acid digested in HNO_3 with H_2O_2 in microwave mineraliser, metal concentrations were determined by ASA or/and ICP-MS) (iii) expression of *AhHMA4*, *LeIRT1*, *LeFER*, *LeFRO* genes in roots and leaves (Real-Time PCR, as a reference gene: *EF1α* was used). The results obtained for the transformed plants were compared to wild-type ones.

RESULTS AND DISCUSSION

Hydroponic experiments

Transgenic tomato expressing *AhHMA4p1::AhHMA4* did not show any distinguishable differences in visual appearance when grown for 23 days on hydroponic cultures (at 1/10 and ½ Knop's medium without and with cadmium). We did not detected the differences in the Zn, Cd and Fe concentration in roots between plants expressing *AhHMA4* and wild-type ones exposed to low and optimal ions concentration in the medium (without and with Cd). In leaves however, the Zn concentration was found to be higher than in the wild-type plants.

Soil experiments

No differences were detected between transgenic and wild-type plants in their appearance until the 53rd day of growth. At later stages, the leaves of transgenic plants began to develop necrotic areas while those of wild-type did not. After prolonged cultivation necrotic areas have occurred also over the leaf blades of wild-type plants. To determinate if the expression of *AhHMA4* modifies the fruit yield and seed production, plants were grown in soil until fruit ripening. It was detected that *AhHMA4* expressing tomato displayed a tendency to more intensive Zn translocation to the shoots when grown both on control and Cd spiked soil. It is not excluded that as a consequence of better shoots supply with Zn the number and biomass of fruits and number of seeds was improved in the transgenic plants compared with wild-type ones. Moreover, in transgenic plants Cd concentrations in the roots was lower, and a tendency to accumulate less Cd in the leaves and fruits was noted. Restriction of Cd concentration was already noted for *AtHMA4* expressing tobacco, and it was referred to the development of the apoplastic barrier within the external layers of tobacco roots (Siemianowski et. al 2013; 2014).

Expression analysies

The expression level of *AhHMA4* in transgenic tomato was similar in the roots of plants growth both on soil and in the hydroponic cultures (both with and without Cd), addition of Cd in medium did not affect the *AhHMA4* expression level. These suggested that the expression of *AhHMA4p1::AhHMA4* in tomato ins not regulated by applied growth conditions.

The Fe concentration was not modified in *AhHMA4p1:AhHMA4* -expressing tomato (as compared with the wild-type). However, the expression of *LeIRT1* (the Fe-deficiency-inducible Fe uptake gene) was differentially regulated in transgenic plants. Its transcript level was enhanced in roots of transformants, primarily when exposed to 1/10 Knop's medium, and to a lesser extent upon ½ Knop's. These indicate, that the *AhHMA4* expression contributed to generation of the Fe deficiency signal, which triggers the expression of *LeIRT1*.

CONCLUSIONS

The expression of *AhHMA4p1::AhHMA4* in tomato seems to be beneficial for fruit and seed development in plants growing in Cd-contaminated soil.

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Effects of Magnesium Supply on Wheat (*Triticum durum*) Grown under Ambient and Elevated Carbon Dioxide Conditions

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INTRODUCTION

The Earth's climate is currently changing at rates incomparable to the pre-industrial era. Elevation of greenhouse gas emissions and more specifically rising of atmospheric carbon dioxide (CO_2) is the main reason of global climate change. According to the IPCC scenarios (IPCC,2007) atmospheric CO_2 concentrations which is currently around 400 µmol mol^{-1} will rise to 530-970 µmol mol^{-1} by the end of this century unless a strict greenhouse gas mitigation is put forward on a global scale. On the other hand, carbon is the main nutrient of higher plants assimilated through the unique photosynthesis process. Therefore, it is a major challenge for plant scientists to understand the responses of plants to the changing climates and elevating CO_2 levels. While there is an ongoing debate whether elevated CO_2 would have a beneficial effect on plant productivity or not, our knowledge on the interactions of other nutrients with elevated CO_2 is still very limited. This study investigates the effects of magnesium (Mg) nutrition on growth and photosynthetic performance of durum wheat plants grown under ambient and elevated carbon dioxide conditions.

METHODS

Durum wheat plants (*Triticum durum*, Saricanak 98) were grown with adequate (1000 µM) and low Mg (75 µM) supply in nutrient solution and under two different CO_2 conditions (ambient: 400 µmol mol^{-1} and elevated: 700 µmol mol^{-1}). When plants were two weeks old gas exchange parameters and chlorophyll content were measured in the second oldest leaves. Specific leaf weight was calculated by the ratio of leaf dry weight to leaf area following image processing of scanned fresh leaf samples.

RESULTS AND DISCUSSION

As expected, low Mg supply significantly reduced biomass production (i.e. shoot+root dry weight) of plants (Fig. 1). Elevated [CO_2] enhanced biomass production in plants supplied with adequate Mg, however it was slightly reduced in the case of low Mg supply. Low Mg also resulted in an increase of leaf specific weight irrespective of the [CO_2] treatments (Fig. 1). The increase in specific weight can be ascribed to the defective sugar transport mechanism observed in Mg deficient plants. It has been reported that Mg has a crucial role in phloem loading of sucrose (Cakmak and Kirkby, 2008; Hermans et al., 2005).

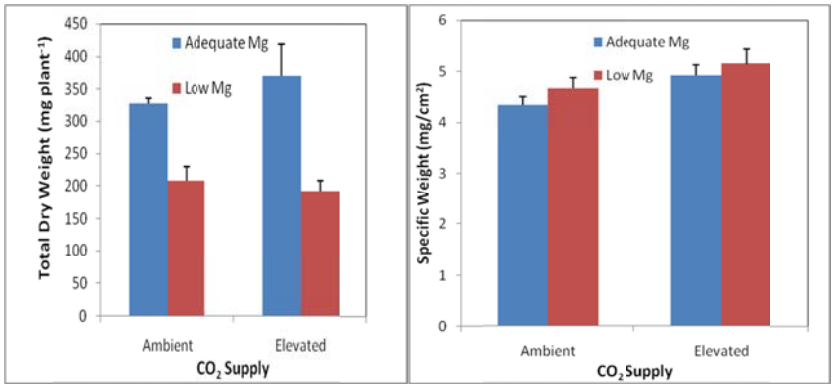

Fig. 1: Biomass production (i.e. shoot+root dry weight) and specific leaf weight of two-weeks-old durum wheat plants grown under adequate (1000 µM) and low Mg (75 µM) supply and ambient (400 µmol mol^{-1}) and elevated (700 µmol mol^{-1}) CO_2 concentrations.

Specific leaf weight was highest in plants grown with low Mg supply and elevated $[CO_2]$, suggesting that sugar accumulation in source leaves can increase as a result of both Mg deficiency and elevated $[CO_2]$ conditions. This findings also consistent with changes in photosynthesis rate of plants as affected by Mg and CO_2 treatments (Figure 2).

In adequate-Mg plants photosynthesis rate was enhanced by elevated $[CO_2]$, however, low-Mg plants lacked such an additive effect brought about by elevated $[CO_2]$ conditions (Fig. 2). This is probably due to the well-known negative feedback effect of sucrose accumulation in leaf cells on the normal functioning of photosynthetic pathway (Long et al., 2004). The increments in leaf specific weight by low-Mg and elevated-$[CO_2]$ further support this effect. Chlorophyll content of plants also resembled changes in net photosynthesis rates. In adequate-Mg plants there was a slight increase by elevated $[CO_2]$, however in low-Mg plants chlorophyll was not affected if not reduced by elevated $[CO_2]$ (Fig. 2).

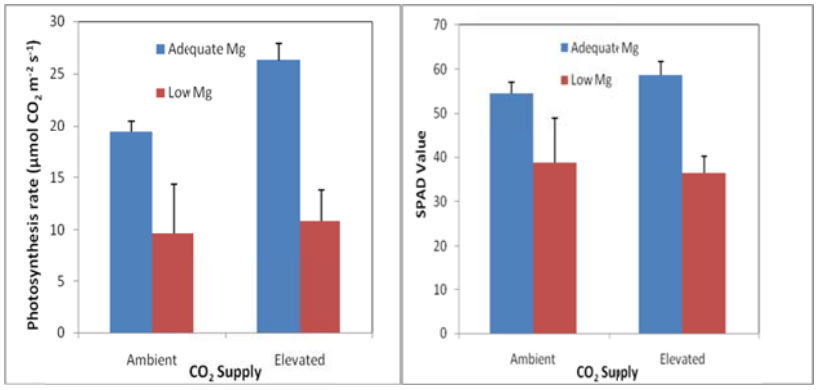

Fig. 2: Photosynthesis rate and chlorophyll concentration (SPAD) of two-weeks-old durum wheat plants grown under adequate (1000 µM) and low Mg (75 µM) supply and ambient (400 µmol mol^{-1}) and elevated (700 µmol mol^{-1}) CO_2 concentrations.

CONCLUSIONS

The possible effects of elevating atmospheric $[CO_2]$ on crop productivity depend on numerous environmental factors including mineral nutrition. Adequate Mg nutrition is required to maintain a high photosynthetic efficiency and a balanced export of photo-assimilates to sink organs (Marschner, 1995; Cakmak and Kirkby, 2008).

The results of this study point out the importance of adequate Mg nutrition under elevated $[CO_2]$. It was shown that reductions in biomass production and photosynthetic efficiency resulting from inadequate Mg nutrition were further exacerbated under elevated $[CO_2]$ conditions. We conclude that nutrition of plants with sufficient Mg can be even more critical under elevated $[CO_2]$ conditions where carbon, the main substrate of photosynthesis is more abundant.

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Modification of Zn, Cd and Fe Accumulation in a Model Plant by Heterologous Expression

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INTRODUCTION

Plants require a range of metal micronutrients such as Zn, Fe, Cu and Mn for growth and development. A key process following uptake from the soil by the root is root to shoot transfer. Molecular and biochemical studies have indicated that it is under tight control and is also influenced by the presence of non-essential metals such as Cd. Understanding the mechanisms involved in the regulation of the underlying processes could contribute to engineering a desired pattern of metal accumulation in particular plant parts. This is key for biofortification.

Zn loading into the xylem plays a crucial role in the control of Zn translocation to the shoot. Recent analysis demonstrated that P1B-type ATPases such as AtHMA2 and AtHMA4 from A. thaliana, and also HvHMA2 from Hordeum vulgare, are responsible for transporting Zn and also Cd from the roots to the shoots (Mills et al., 2003; Hussain et al., 2004; Mills et al., 2012). They contrbute to metal xylem loading by driving the efflux to the apoplast.

Taking into account the role HMA2 and HMA4 play in the translocation of Zn (Cd) to the shoot, there were attempts to use these genes to engineer enhanced Zn level in the aerial plant parts for phytoremediation / biofortification. Although for these proteins both Zn and Cd are substrates, unexpectedly it was detected that expression of AtHMA4 in tobacco reduced Cd accumulation (Siemianowski et al., 2011, 2013, 2014).

In this research, it was analysed whether the heterologous expression of HvHMA2 from Hordeum vulgare in tobacco under the CaMv35S promoter overcomes the root-to-shoot Zn translocation barrier thus increases its concentration in aerial parts. Moreover, modification of Cd accumulation was great concern. Important part of the study was evaluation of the dependance of the Zn and Cd accumulation pattern on metals supply in transgenic and wild-type plants.

Tobacco was used as a model plant as it is easy to transform and regenerate.

METHODS

Tobacco (Nicotiana tabacum cv. Xanthi) wild-type and four homozygous lines expressing HvHMA2 from Hordeum vulgare L. under control of the double CaMV35S promoter (Mills et al., 2012; Barabasz et al., 2013) were used as an experimental material.

Evaluation of Zn and Cd Tolerance and Accumulation: For the determination of the modification of Zn and Cd accumulation and tolerance due to the expression of HvHMA2, four-week old seedlings were exposed in hydroponics for 10 days: (i) to 1, 10 µM or 100 µM Zn; (ii) to 0.25 and 5 µM Cd. The tolerance to zinc and cadmium were evaluated based on visual assessment of plant health and dry biomass yield. The Zn, Fe and Cd concentrations were determined in the roots and the shoots by AAS.

Apoplastic Fluid Analysis: The Zn concentration in the apoplastic fluid of plants exposed to the control cnditions and to 100 µM Zn for two days, were determined according to Dannel et al. (1993) and Lòpez-Millán et al. (2000) with some modifications (Barabasz et al., 2012).

Transient Expression of HvHMA2-GFP in Tobacco Leaves: The construct fusing a GFP coding sequence to the N-terminus of HvHMA2 under control of double CaMV35S promoter was prepared by Mills et al. (2012). Here it was used for transient expression of HvHMA2 in leaves of tobacco. After 3 days from the infiltration of leaves with Agrobacterium tumefaciens carrying the HvHMA2-GFP construct, the confocal microscope analysis was performed.

RESULTS AND DISCUSSION

Our results demonstrate that in transgenic tobacco, HvHMA2 protein localizes to the plasmolemma, thus its export activity might result in overloading of the apoplast with Zn. Therefore, we compared the Zn concentration in the apoplastic fluid between the wild-type and transgenic plants. It was shown that the Zn concentration in the apoplast of the AtHMA4-expressing plants was two-fold higher than in the non-transgenic ones. Thus, the Zn status at the cellular level was modified, which likely contributed to the generation of the phenotype of transgenic plants. Results indicate that ectopic expression of the export protein HvHMA2 in tobacco interferes with its endogenous metal Zn-Cd-Fe cross-homeostasis, inducing internal mechanisms regulating metal uptake and tolerance. Moreover, transformation with HvHMA2 did not produce one unique pattern of Zn and Cd accumulation; instead, it depended on external metal supply. Thus Zn and Cd root-to-shoot translocation was facilitated, however not at all applied Zn/Cd concentrations.

Overloading of the apoplast with Zn, thus alteration of the Zn status at the cellular level, likely contributes to induction of the endogenous tobacco mechanisms responsible for the detected overall decrease in metal uptake.The Zn uptake was restricted in HvHMA2-transformed plants grown at 10 µM, but due to its efficient translocation to aerial plant parts, the level in the shoot did not decrease. Upon exposure to high toxic Zn concentraion (100 µM Zn), despite the lower than in WT levels of Zn in the shoot of transgenic plants, HvHMA2 transformants showed increased Zn-sensitivity.

Moreover, although there is no evidence that HvHMA2 transports Fe, expression of HvHMA2 in tobacco interfered with Fe metabolism and Fe root-to-shoot translocation and accumulation of this metal were modified in HvHMA2-transformants in a Zn- and Cd-concentration dependent manner.

CONCLUSIONSC

Results indicate consequences of the transformation not related to the usual physiological function of HvHMA2 in barley, which have to be taken into account when planning expression of metal export proteins to modify metal accumulation.

ACKNOWLEDGEMENTS

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Possible Mg, Fe, Mn and Zn Availability from Maize Lines belonging to different Heterotic Groups

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INTRODUCTION

In the past, crop breeding and production had been focused on increasing yield and maintaining food and feed production stability. Parallel with that, concentration of essential macro and micro nutrients have been decreased. From the nutritional point of view starch, protein and oil content mainly defined maize quality characteristics. Agricultural strategies for enhancing micronutrient concentrations in grain were recently being assessed as a sustainable, long-term solution. Among these strategies, plant breeding appears to be the most sustainable and cost-effective approach. Considerable genetic variation in maize can be utilizable for sustainable biofortification strategies. They include not only increase of micronutrient concentrations in grain, but also factors which decrease or increase availability of present micronutrients, such as phytate, β-carotene and others. Present breeding programs include phytate lowering (factor which obstruct absorption) in grain, as well as β-carotene increase (factor which contributes to better absorption) in grain and other edible parts of plants.

The aim of this study was to investigate grain chemical composition of maize inbred lines, belonging to different heterotic groups and to define their nutritional quality from the point of view of Mg, Fe, Mn and Zn availability.

METHODS

Grains of 66 maize inbred lines with different genetic background from MRI breeding program were used. The 28 inbred lines belong to BSSS germplasm, 12 to BSSS independent source germplasm, 16 to Lancaster germplasm and 10 to European (mostly local) germplasm. Microelements were measured by Inductively Coupled Plasma - Optical Emission Spectrometry after wet digestion with HNO3+HClO4. Inorganic (Pi) and phytic phosphorus (Pphy) were determined calorimetrically from 5% TCA extract (Dragicevic et al., 2011) and β-carotene with AACC (1995) procedure.

RESULTS AND DISCUSSION

Pphy content in grain ranged 2.3-3.8 g kg-1, with the lowest average content noticed in BSSS group and the highest variation obtained in Lancaster group (11%). Pi ranged 0.32-0.80 g kg-1, with the highest average content in European group and the highest variation obtained in BSSS independent group (25%). β-carotene content ranged 1.9-17.5 g kg-1, with the highest average content in BSSS independent source and the highest variation obtained in European group (43%). Mg content varied in range 587.8-973.1 mg kg-1, with relatively higher average values present in Lancaster group and minor variations between heterotic groups (12-13%). Fe content varied in range 2.3-30.5 mg kg-1, with the highest value and variation achieved in BSSS group (41%). Mn content varied in range 2.8-10.1 mg kg-1, with the highest average value in BSSS independent source group and the highest variation between genotypes in BSSS group (32%). Zn content was in the range 8.3-35.8 mg kg-1, with the highest average values and variations obtained in BSSS independent source group (40%). It was also characteristic for BSSS group that higher values of β-carotene, Mg, Mn and Zn were obtained in seeds with higher weight.

Regression analysis stressed significant and positive interdependence between seed weight and Mg and Mn content in grain of all heterotic groups, except for BSSS independent source group, where this correlation was negative. Significantly negative interdependence between Pphy and Fe content was noticed in BSSS and European group, while interdependence between Pphy and Mg was positive in BSSS

independent source and European group. From this point, these three heterotic groups and particularly European group could be considered as source of genotypes with increased Fe availability. β-carotene was positively correlated with Mn content in grain of Lancaster and European groups. According to Principal Component Analysis, Fe, Mg, Zn and Pphy mostly contributed to PC1 axis, as well as Pi, Mn and β-carotene to PC2 axis, indicating different patterns that affect accumulation of Mg, Fe, Mn, Zn, as well as β-carotene and Mn content. This could indicate that breeding programs that work on Pphy (which restrain absorption of mineral nutrients) decrease in maize grain could not obtain β-carotene increase (which enables absorption of mineral nutrients) in the same material.

Molar ratios between Pphy, Pi, β-carotene and examined micronutrients varied in broad range, but we noticed the lowest Pphy/Pi, Pphy/β-carotene, Pphy/Mg, Pphy/Fe Pphy/Mn and Pphy/Zn ratios in lower weight grains of all heterotic groups (1000 grains weight < 250 g). The exception was European group, where none constancy was recorded. This could be linked to high heterogeneity present in this heterotic group. However, relatively favourable ratios between Pphy, β-carotene and examined micronutrients are present in grains, moderate in Pphy (about 3 g kg-1), that could linger standard breeding for Pphy lowering.

CONCLUSIONS

Among analysed genotypes, BSSS heterotic group was characterised with the lowest ratios between Pphy, Pi, β-carotene and micronutrients, which could mark it as the most desirable group in breeding programs for Pphy lowering, parallel with increase of micronutrients' content in maize grain. This is supported by tendency that decreased Pphy content was mainly connected with increased Fe and Mg content. It is also important to underline that connection between micronutrient concentration and grain weight could provide high yielding potential together with increased micronutrient level. On the other hand, strategy for selection of high β-carotene maize could be linked with higher Mn content in grain. From this point, European heterotic group could be interesting, because of the relatively high β-carotene level in grain. Together with the lowest Pphy/β-carotene value in some genotypes from this group, it could mark them as possible source for increased Mn availability. Further investigations could provide availability of examined micronutrients, particularly from hybrids derived from lines with lowest ratios between examined factors.

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Towards Construction of the Zn Homeostasis Network in *Arabidopsis thaliana* and *Noccaea caerulescens*

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INTRODUCTION

The daily diet of the global human population is based mainly on cereals like rice, wheat, and maize. When these crops are grown in Zn deficient soils, the grains present very low Zn concentrations and the population has high chances to present Zn deficiency (Cakmak, 2008). The WHO has determined that around 3 billon people suffer from Zn deficiency, considering it a major public health problem.

Plant Zn homeostasis includes a wide range of processes in charge to maintain the balance of Zn concentrations to fulfil their physiological requirements and avoid toxicity according to the external supply (Sinclair and Krämer, 2012). Zn homeostasis is a very tight controlled process driven by metal transporters, chelators, transcriptional factors, post transcriptional regulators and translational regulators, during Zn uptake, translocation, storage and remobilization (Vreugdenhil et al., 2004; Sinclair and Krämer, 2012). Zn transport can be considered as a series of ligand exchange reactions (Blindauer and Schmid, 2010). Low molecular-weight ligands as citrate, oxalate, malate, phosphate, nicotianamine, glutathione, phytochelatins, and histidine are Zn ligands (Blindauer and Schmid, 2010; Sinclair and Krämer, 2012). For instance, nicotianamine (NA) is a high affinity metal chelator involved in the symplastic Zn mobility between cells and in long distance transport in the phloem (Salt et al., 1999).

Different Zn transporters have been identified in *A. thaliana* which are involved in Zn uptake from the soil by the root, radial movement towards the xylem vessels, cortex and stele, distribution to the shoot of the plant by xylem transport, and storage (Grotz et al., 1998; Sinclair and Krämer, 2012). The ZIP (ZRT-, IRT-like Proteins) family transports cations like Zn, Cd, Fe, Mn, and are mainly related with cellular Zn uptake in roots (Grotz et al., 1998; Vreugdenhil et al., 2004). The HMA (Heavy Metal ATPases) family is involved in xylem loading. *HMA2* and *HMA4* are mainly expressed in the vascular tissues and their activity is related with the Zn translocation from root to shoot (Hussain et al., 2004; Hanikenne et al., 2008). The CDF (Cation Diffusion Facilitator) family includes heavy metal efflux transporters of the divalent cations Zn, Fe, Cd, Co, Ni and Mn (Dräger et al., 2004). A member of this family is MTP1 (Metal Transport Protein) most likely responsible of sequestering the excess of intracellular Zn into the vacuoles (Kobae et al., 2004). Other Zn transporters also involved in Zn homeostasis are YSL (Yellow Stripe-Like), PCR (Plant Cadmium Resistance), NRAMP (Natural Resistance-Associated Macrophage Protein), FRD3 (Ferric Reductase Defective 3) (Pineau et al., 2012), ABC, MFS (Major Facilitator Superfamily), and ZIF1 (Zinc-Induced Facilitator 1) (Haydon and Cobbett, 2007).

The transcription factors bZIP19 and bZIP23 are involved in controlling the initial Zn deficiency response. Among the targets of these partially redundant transcription factors are eight members of the ZIP family and two *NAS* genes which are upregulated under Zn deficiency (Assunção et al., 2010).

Although a lot of work has been done to understand the molecular control of Zn homeostasis by studying Zn transporters, more functional evidence is needed to fully understand their functions (Sinclair and Krämer, 2012). Information like tissue expression, cellular localization and Zn binding properties is still missing for many of these proteins. In addition, intracellular Zn movement likely involves a series of ligand exchange reactions (Blindauer and Schmid, 2010), but limited information is available about how these exchange reactions occur, at which points of the transport process (Verbruggen et al., 2009) and what the rate limiting steps are during Zn remobilization (Blindauer and Schmid, 2010). In my project, I will further study the genes involve in the Zn fluxes in *A. thaliana* and *N. caerulescens* to understand the gene regulatory network of Zn in plants.

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Water Rich in Iron to Irrigate Lettuce Crop

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INTRODUCTION

The importance of iron lies in the fact that it is believed to be the tenth most abundant element in the universe and is the most abundant element that making up the Earth. This element can be found in water, soil, food and plants and is essential to almost living things, from microorganisms to humans.

Since iron is an essential and common in and for the life, is very usual to find concentrations of it in domestic and industrial effluents and surface water.

Sometimes iron presence is suitable, other time it is neglected.

In some case we study how to remove iron from soil, water and air, in other case we are focused how to add it.

Agronomic plants sometime are too rich in metals, because growth in contaminated soil, other time too poor, because of the the soil shortage of this element.

Iron deficiency cause healthy problems; natural and chemical methods exist to avoid diseases.

Is iron a contaminant element or a beneficial element?

The difference is in the concentration and in the form.

Sophisticate biotechnology exist to process agronomic plant. This research aims to demonstrate that water contaminated by iron, but under control can be utilized positively (with benefit) to irrigate the lettuce crop in order to promote a bioaccumulation.

We describe a green technology, a constructed wetland, used to remove excess of iron from water and how to use the outflow (still containing a suitable quantity of iron).

METHODS

The phytodepuration is considered a green technology and has been widely used for domestic, industrial, zoo-technical and mining wastewater, but with removal efficiency no more than 80/90 %. After the constructed wetland treatment, comes afloat the question on whether system is effective removing and what to do with the water leaving the system

The phytodepuration pilot plant uses first a vertical flow system (SFS-v) with 2 tanks in parallel (A & B)and then the horizontal flow system (SFS-h) with a single tank (S). The first step in the depuration plant is pumping into the vertical tanks. *Typha latifolia*, *Juncus* and *Aster tripolium* are planted in the pilot plant. Concentration of Iron loaded into the phytodepuration system was 50mg/l.

The water treated by the system was used as irrigation source for lettuce crops.

RESULTS AND DISCUSSION

Chemical analysis show:

- the system removal efficiency is equal to 65%;
- the water is suitable for irrigation;
- the accumulation in lettuce crop do not exeed ecotoxicological limits.

The phytodepuration could be a valid technology to be used anytime we don't need the complete contaminant removal.

Table 1. Iron concentration in the water samples during the constructed wetland treatment

	Tank Fe (ppm)		
Date sampling	SFS-v (A)	SFS-v (B)	SFS-h (S)
1 april	0,463	0,188	0,030
1 may	0,098	0,070	0,073
29 may	0,037	0,027	0,016
1 july	0,053	0,040	0,033
29 july	0,081	0,018	0,041
1 october	0,059	0,046	0,062

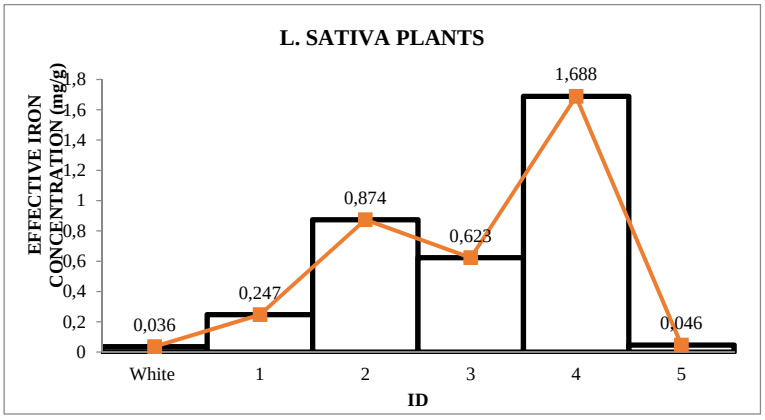

Fig. 1: Iron concentration in *L.sativa*

CONCLUSIONS

It would be useful to consider that the irrigation of lettuce crops with water exiting the phytodepuration system could be beneficial in "fortified food". In this case it is necessary to analyse what is the optimal point of iron fortification of lettuce and where presents the best conditions to allow greatest gain in nutritional benefits and environmental conservation.

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Zn and Fe Biofortification in *Triticum aestivum* L.– A Field Study

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INTRODUCTION

Zinc and Fe biofortification of staple foods, namely *Triticum aestivum* L., might overcome nutrient deficiencies in humans. Yet, that might limit the nutritional and technological value of the flour. In this context, the we aim at assessing the potential implications of wheat Zn and Fe biofortification on flour quality parameters, yield and photosynthetic performance under field conditions.

METHODS

Parental (F0) and F2 generation seeds of *Triticum aestivum* L. cv Roxo previously biofortified in a walk-in growth chamber, with standard (s) and 5 fold higher levels of macro and micronutrients (5s), were sown in trails in irrigated field conditions, between December 2012 (sowing) and July 2013 (harvesting) in Elvas, Portugal. Bread wheat (cv Roxo) was submitted to two levels and type of fertilization: F3 (s), and F3 (5s) - with N (urea 46%); F3 (s) and F3 (5s) - N (urea 46%) + Zn (11g Zn/ha) + Fe (200g Fe/ha), biofortified plants, applied in three different phases of the growth cycle (tillering, heading and grain milk stage). Before sowing all treatments were fertilized with P-K-N 14-25-8 (200kg/ha) and Zn SO_3 (50kg Zn/ha). F4 (s) and F4 (s/s) were obtained from the F3 (s) plants, whereas F4 (5s) and F4 (5s/s) were obtained from F3 (5s). Weeds and diseases were controlled using agrochemicals following the manufacturer´s recommendations for application.

Zn and Fe were measured after incineration at *ca*. 550^{o}C followed by nitric acid digestion (Unican model 939 absorption unit). A LI-6400 Portable Photosynthesis System (LI-COR, USA) was used for leaf gas exchange measurements, whereas Chl *a* fluorescence was evaluated with a PAM-2000 system (H. Walz, Germany). Wheat grain composition was evaluated by protein (ISO 20483:2013), ash (NP 519.1993) and lipid contents. Lipids were extracted in a chloroform/methanol/water mixture (1:1:1, v/v/v), evaporated and saponified (0.5 M NaOH in methanol). The fatty acids were methylated with BF_3 and analyzed by gas liquid chromatography (UNICAM 610 gas chromatograph, UK). The wheat technological quality was evaluated by SDS sedimentation test (Dick and Quick, 1983) and a rheological test through the micro-alveograph method (ISO 27971, 2008, adapted).

RESULTS AND DISCUSSION

The amounts of Zn and Fe in the biofortified crops did not vary significantly between F3s and 5s, or between F3 and F4 treatments, yet these nutrients increased *ca*. 5 and 1.6 fold, respectively, in relation to F0(s) (Table 1). To ensure that wheat plants did not surpass the threshold of toxicity, during grain filling of F4 treatments, photosynthetic related parameters were monitored (Fig 1, Table 2), being maintained quite high, without negative influence of high nutrient availability levels. In this context, the evaluation of the technological/nutritional properties of the crops (Table 3) revealed that gluten strength evaluated by SDS test did not vary significantly among the biofortification treatments. Moreover, W value (energy required to deform the dough bubble until it bursts in alveograph test) decreased significantly in biofortified crops of all treatments (relatively to F3 (s)). Additionally, P/L ratio (balance between dough tenacity and extensibility) was particularly higher when the N plus Zn and Fe was applied (F4 (s/s) and F4 (5s/s)). Still, the yield strongly augmented in F4 (s, s/s, 5s, 5s/s) when compared with F3 (s) and F3 (5s), but the levels of protein decreased in all the F4 treatments (Table 3). Concomitantly, amongst the F4 treatments, the levels of individual and total fatty acids (TFA) did not vary significantly (Table 4).

Table 1. Zn and Fe contents in the seeds of *T. aestivum*. Values are the mean (n=5) .

	F0(s)	F3 (s)	F3 (5s)	F4 (s)	F4 (s/s)	F4 (5s)	F4 (5s/s)
Zn (ppm dw)	15.2	81.1	76.6	79.1	79.3	75.7	76.3
Fe (ppm dw)	25.6	39.9	44.9	41.2	36.2	48.3	47.3

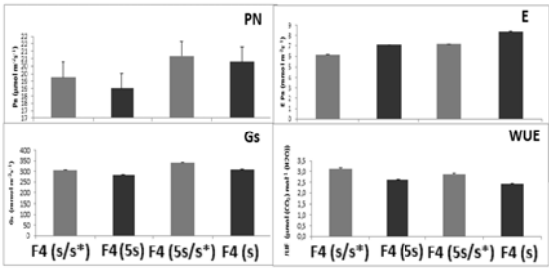

Fig. 1. Gas exchange measurements. Values are the mean±SE (n=5)

Table 2. Fluorescence parameters. Values are the mean (n=4).

	F_t	ETR	Yield	q_P	F_m'	F_o'	F_v'/F_m'
F4 (s)	0.19	259	0.513	0.88	0.40	0.17	0.580
F4 (s/s*)	0.22	235	0.467	0.85	0.42	0.19	0.547
F4 (5s)	0.21	230	0.456	0.81	0.39	0.17	0.559
F4 (5s/s*)	0.23	233	0.463	0.80	0.43	0.18	0.578

Table 3. Prodution parameters of F3 and F4 crops.

	Protein (%)	SDS (mm)	Ash (%)	W ($E^{-4}J$)	P/L	Yield (kg/ha)	Test weight kg/hl	Thousands kernel weight (g)
F3 (s)	17.3	59	2.15	488	1.22	3535	78.97	39.77
F3 (5s)	16.6	64	2.19	333	1.46	3288	79.61	40.01
F4 (s)	12.7	57	1.47	259	1.05	5366	85.41	44.75
F4 (s/s*)	11.0	55	1.60	172	3.10	4943	86.06	48.47
F4 (5s)	11.9	57	1.47	171	1.79	4977	85.63	44.78
F4 (5s/s*)	12.3	59	1.56	186	2.94	4867	85.25	45.33

Table 4. Grain fatty acids composition (Mean±SE, n=3).

	Fatty Acids(mol %)						TFA	DBI
	C16:0	C18:0	C18:1*c+t*	C18:2	C18:3	Less Rep.	(mg/g dw)	
F4 (s)	18.3±0.1	1.1±0.0	14.8±0.1	60.5±0.1	4.2±0.0	1.2±0.0	6.3±0.1	7.2±0.1
F4 (s/s)	19.2±0.4	1.1±0.0	15.1±0.0	59.4±0.3	4.1±0.0	1.2±0.0	5.5±0.2	6.8±0.2
F4 (5s)	18.3±0.2	1.0±0.0	15.1±0.1	60.2±0.3	4.2±0.0	1.2±0.0	6.6±0.1	7.2±0.1
F4 (5s/s)	18.7±0.3	1.1±0.0	15.0±0.1	59.8±0.4	4.1±0.0	1.3±0.1	5.9±0.2	7.0±0.2

CONCLUSIONS

Zn and Fe biofortification of *T. aestivum* grains can reach about 5 and 1.6 fold increase, respectively, following the experimental design of this study, without attain the threshold of plants toxicity to photosynthetic related parameters and yield. Moreover, the technological and nutritional properties, although varying, do not trigger significant limitations to human health.

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12 Year Experience on Crop Growing in Almadén Soil

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INTRODUCTION

Almadén (Ciudad Real, Spain) has been considered the largest and oldest mercury (Hg) mining area and its production represented above one third of the total Hg extracted in the world. Thus, during centuries, the economy of Almadén area depended on mining activities. However, in the last decade, after ceasing the extractive activities in 2002, the entire region has undergone an economic depression. In order to mitigate this situation, it is necessary to find socio-economical alternatives for the local population, including the implementation of alternative land uses like the agricultural one. In this context the aim of this twelve year work experience (Millán *et al.*, 2002 and 2013) has been to study the behaviour of Hg in the soil-plant system within this area where Hg mining had a profound impact. Thus, several typical crops were cultivated in order to give a scientific basis for elaborating a list of recommendations on sustainable and safe agricultural land use, without risks for human or animal health, according to current international legislation.

METHODS

The experimetal work was performed at different scales, including the cultivation of crops in pots and lysimeters. Lysimeter experiments make possible to work under close-to-real conditions due to the advantage of preserving the soil horizon characteristics. The cultivations were white lupin (4 yields in lysimeter), chickpea (1 yield in pots; 1 yield in lysimeter), common vetch (4 yields in pots; 4 yields in lysimeter), barley (4 yields in lysimeter), lentil (1 yield in pots; 4 yields in lysimeter), lavender (3 yields in pots), eggplant (3 yields in pots) and Swiss chard (2 yields in lysimeter).

Plant pots were filled with a mixture of soil from Almadén, perlite and sand on equal proportion. The physico-chemical characterization of soil was described by Sierra (2009, 2008a, 2008b). The lysimeter experiments used in this work consist of a cubic meter of an unaltered soil monolith within a metallic structure, from the same place where pot soil was collected. The physico-chemical characterization of soil is described by Schmid *et al.* 2004 and Sierra *et al.* (2011, 2008b).

Plants were harvested at maturity. To study the distribution of Hg through the plant, each plant sample was divided in roots, stems, leaves, fruits/seeds and pods. Samples were placed in individual beakers and rinsed several times to remove external contamination. Afterwards, all samples were dried and grinded. Mercury concentration in soil and plant samples was determined using an Advanced Mercury Analyser (AMA-254).

RESULTS AND DISCUSSION

Total Hg concentration in pot substratum varied between 2.1 ± 0.7 mg kg-1 and 10.1 ± 0.5 mg kg^{-1}, while, total Hg concentration in lysimeter soil varied between 13.8 ± 2.0 mg kg^{-1} and 28.9 ± 0.4 mg kg^{-1}. In both cases, less than 0.3 % of Hg is easily available for plants. Furthermore, in all studied crops (grown in pots and lysimeters), Hg distribution through the plant at maturity followed the following relation: $[Hg]_{roots} > [Hg]_{leaves} > [Hg]_{stems} > [Hg]_{pods} > [Hg]_{seeds/fruits.}$

Regarding a safe consumption in both scales, the table 1 shows that the Hg concentration in the forage crop seeds were lower than the limit established by the European legislation (0.1 mg kg^{-1}) thus this plant part could be consumed by animals. However, the consumption of the obtained fodder by animals would not be advisable. In the case of the crops that can be consumed by humans cultivated in these soils, their consumption does not present a risk for the human health.

Table 1. Summary of the safety consume of different crops. * It would be advisable to be aware of using it for feeding animals.

	Could the crop be safely consumed by animals?		Maximum ration of edible grain that can be consumed by a human consideringtaking into account as the only Hg contribution to the diet (FAO-WHO, 2011)			
Crop	Fodder	Seed	Grain or fruit (kg week^{-1})	Raw leaf (g day^{-1})	Young shoot (kg week^{-1})	Infusion (l day-1)
White lupin	YES*	YES	7.7	-	-	-
Chickpea	NO	YES	19.6 - 38.5	-	1.8 - 2.2	-
Common vetch	NO	YES	-	-	-	-
Barley	NO	YES*	0.9 – 49.0	-	-	-
Lentil	-	-	15.4 – 35.7	-	-	-
Lavender	-	-	-	-	-	3.7 – 68.4
Eggplant	-	-	1.3 – 14.3	-	-	-
Swiss chard	-	-	-	30 – 350	-	-

CONCLUSIONS

According to the obtained results, Hg accumulation in vegetative organs represents a higher potential risk for animal consumption of all the crops tested, while seeds and fruits can be used, both for human and animal consumption, according to international legislation. These data can be useful in a complete toxicological risk assessment, where other potential sources that contribute to the Hg intake pathway should also be taken into account.

The present results provide realistic information to be integrate in a scientific database to be use in future changes for land use recommendations in the Almadén mining area where different recuperation strategies are being evaluated.

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Influence of Fungal Endophytes on the Uptake and Accumulation of Trace Elements into *Ornithopus compressus* Biomass

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INTRODUCTION

Yellow serradella (*Ornithopus compressus* L.), which has a Mediterranean basis, is a very common pasture species in the Spanish grasslands. Due to its high quality forage, *O. compressus* is very used for animal feeding. Many factors, such as soil, climate, plant phenology, diseases, etc, could affect the forage nutritive value and mineral content. Among such factors, fungal endophytes have been shown to produce changes on the mineral status in other pasture species, such as *Festuca rubra* L. and *F. arundinacea* Schreb (Zabalgogeazcoa et al., 2006). The objective of the study was to evaluate the effect of four fungal endophytes on the mineral status of *O. compressus* forage.

METHODS

Four fungal endophytes, previously isolated from pasture species and identified in our lab as *Sordaria fimicola* (Rob. ex Desm.) Ces. & De Not. (Code E071), *Embellisia leptinellae* Simmons & Hill (E138), *Epicoccum nigrum* Link (E631) and *Sporormiella pilosa* (Cain) Ahmed & Cain (E636), were used to be inoculated in the experiment. Two months before the inoculations, fungi were grown in culture medium PDB (Potato Dextrose Broth) to obtain enough inoculum. In November 2012, *O. compressus* seeds were surface sterilized and sown (ten seeds per pot) in 6x6x7 mm pots containing peat and vermiculite (1/1 w/w), placed in a greenhouse and watered every 2-3 days until field capacity. Two monts later, plants were wounded and inoculated with a hand sprayer after mycelia and culture medium blending. Inoculation was repeated two weeks later. Five replicates per treatment were used. Five aditional pots were inoculed with just culture medium to be used like control. After inoculations, plants were mantained under greenhouse conditions.

After two months, aerial biomass was cut and carried to the lab for processing. The whole experiment was completely repeated three weeks later (two trials). Samples were air dried and sent to the Ionomics Service of CSIC (Spanish High Centre for Science and Research) for mineral determinations. After a digestion with HNO_3/H_2O_2 in UltraClave Microwave Milestone, samples were proccesed by means of inductively coupled plasma optical emission spectrometry (ICP-OES) to obtain the concentrations of the following minerals: Al, B, Fe, Cu, Mn, Mo, Se, and Zn. Such determinations were also performed on four soil substrate samples. Data were statistically analysed by analysis of variance and multiple comparison tests (Fisher LSD test).

RESULTS AND DISCUSSION

In the soil substrate the concentration of the trace elements evaluated were (mean $\pm$ standard error): 532.9 ± 37.1 mg Al kg^{-1} soil, 3.2 ± 0.2 mg B kg^{-1} soil, 5.2 ± 0.6 mg Cu kg^{-1} soil, 436.2 ± 85.9 mg Fe kg^{-1} soil, 26.6 ± 2.5 mg Mn kg^{-1} soil, <0.1 mg Mo kg^{-1} soil, <0.1 mg Se kg^{-1} soil, and 6.86 ± 1.39 mg Zn kg^{-1} soil. Anova showed that the inoculation with an endophyte affected significantly the concentration into plants of B, Cu, Mo and Zn (Table 1). Plants inoculated with *Embellisia leptinellae* (E138) produce a forage with higher concentration of B and Cu (more than 30% and 44%, respectively) than controls (Table 2). Boron is an essential nutrient for plants but not for animals, and the concentration found in the controls might be higher than that considered deficient (Hosking et al., 1986). Copper, however, is an essential trace element, being involved in at least 10 enzymes which catalyze oxidase type reactions in both plants and animals. Considering that sheeps need 6 mg Cu kg^{-1} dry matter, and cattle 7 mg Cu kg^{-1} dry matter to cover their requirements (Hosking et al., 1986), the inoculation of this pasture species with such endophyte, may accomplish such concentrations. On the other hand, plants inoculated with *Sporormiella pilosa* (E636) or *Epicoccum nigrum* (E631) produced forage with a concentration of Mo and

Zn higher (around 140% and 46%, respectively) than in controls (Table 2). Molybdenum and Zn are essential elements for both plants and animals. The levels obtained in the pasture, even in the controls, should be enough to reach animal requirements (Hosking et al., 1986).

Table 1. Summary of two-way ANOVA for each trace element evaluated. DF. degree of freedom; F values, including the level of significance (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.), are shown in the rest of the columns.

Source	DF	Al	B	Cu	Fe	Mn	Mo	Se	Zn
Endophyte	4	1.38	2.87*	3.98*	2.64	2.12	6.22**	1.77	2.86*
Trial	4	5.16*	2.15	9.34**	27.78***	4.19	2.99	2.83	8.33**
Endop*trial	4	0.51	0.41	0.16	0.53	0.93	0.21	0.41	0.31

Table 2. Concentrations (mean ± standard error) of each trace element in the biomass obtained from plants inoculated with each endophyte. $\bar{x}$: Average obtained from all the treatments.

	Control	E071	E138	E631	E636	$\bar{x}$
Al (mg kg^{-1})	91.5 ± 14.3	74.2 ± 8.3	105.6 ± 22.8	66.5 ± 28.8	48.2 ± 6.9	77.20 ± 9.1
B (mg kg^{-1})	23.4 ± 2.0 b	22.0 ± 3.2 b	30.6 ± 1.2 a	27.6 ± 0.7 ab	26.5 ± 0.6 ab	26.01 ± 1.0
Cu (mg kg^{-1})	5.0 ± 0.5 c	5.6 ± 0.7 bc	7.2 ± 0.3 a	6.1 ± 0.4 abc	7.0 ± 0.3 ab	6.17 ± 0.3
Fe (mg kg^{-1})	112.5 ± 14.5	98.5 ± 13.1	147.6 ± 22.3	96.6 ± 25.2	91.1 ± 13.3	109.27 ± 9.2
Mn (mg kg^{-1})	223.8 ± 24.0	237.9 ± 46.4	318.3 ± 7.3	262.6 ± 16.3	278.1 ± 5.3	264.15 ± 12.9
Mo (mg kg^{-1})	1.05 ± 0.3 c	1.46 ± 0.3 bc	2.18 ± 0.2 ab	2.36 ± 0.2 a	2.55 ± 0.1 a	1.92 ± 0.2
Se (mg kg^{-1})	0.52 ± 0.1	0.48 ± 0.1	0.84 ± 0.1	0.68 ± 0.1	0.57 ± 0.1	0.62 ± 0.1
Zn (mg kg^{-1})	66.0 ± 9.6 c	70.1 ± 13.5 bc	90.8 ± 4.3 abc	94.2 ± 7.2 ab	96.4 ± 4.3 a	83.49 ± 4.6

Averages in the same row, with different lowercase letters mean significant effect of endophyte ($p \leq 0.05$) according to LSD test. When letters do not appear, differences were not significant according to ANOVA.

CONCLUSIONS

As several of the fungal endophytes evaluated, such as *Embellisia leptinellae*, or *Sporormiella pilosa* were able to increase the concentration of some important trace elements, like B, Cu, Mo and Zn, the plant inoculation could be a suitable strategy to deal with potential nutrient deficiencies in forage.

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Does Biofortification with Fe affects Antioxidant Activity and Nutritional Quality of Lettuce?

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INTRODUCTION

Food products with increased nutritive value are response to consumers new expectations. Therefore vegetables enriched with essential elements and/or antioxidative compounds are receiving growing attention (Blasco *et al*. 2008). Critical parameters characterizing quality of functional food, besides elements content and biomass accumulation, is level of ROS and antioxidants, as external source of these compounds may affects human health (Lobo *et al*. 2010). In this work an attempt was made to evaluate effects of biofortification with Fe on Romaine lettuce (*Lactuca sativa* L. var. *longifolia* Lam.), head lettuce (*Lactuca sativa* L. var. capitata) and endive (*Cichorium endivia* L.) (i) biomass accumulation, (ii) levels of ROS, (iii) activity of anti-oxidative enzymes and (iv) content of element.

METHODS

The study objects were plants of Romaine lettuce (*cv*. 'Amadeusz'), head lettuce (*cv*. 'Omega') and endive (*cv*. 'Burundi'). Plants were grown in hydroponic culture in spring for 6 weeks at pH 6.0-6.2, photoperiod 10/14 h day/night and temperature 20/14°C. Fe was applied in the last 4 weeks of cultivation in a readily available form at concentrations of 12 (control), 24, 36 and 48 mg dm^{-3}. Levels of anion-radical ($O_2^{\bullet -}$), hydroxyl radical (OH°), activity of ascorbate peroxidase (APX) and catalase (CAT) were measured spectrophotometrically at wave lengths of 580, 540, 240 and 290 nm respectively. Ions content was recorded with atomic absorption spectroscopy.

RESULTS

Effect of Fe on biomass accumulation in the aboveground parts, both fresh weight and dry matter, of lettuce and endive plants was negative (Table 1). The greatest reduction was recorded in 'Amadeusz' and lowest in 'Omega' plants (Table 1).

Table 1. Fresh weight and dry matter of leaves of lettuce and endive plants grown in nutrient solution enriched with Fe ions. Data are mean ±SE, n = 5.

Cultivar	Measured parameter	Fe concentration (mg dm^{-3})			
		Control (12)	24	36	48
'Amadeusz'	Fresh weight (g)	186.3	99.9	91.2	17.4
	Dry matter (g)	13.6	8.6	5.9	2.5
'Burundi'	Fresh weight (g)	130.1	118.5	66.1	47.4
	Dry matter (g)	8.3	7.5	4.8	4.2
'Omega'	Fresh weight (g)	192.9	180.4	126.2	19.4
	Dry matter (g)	8.3	9.5	7.3	2.5

Levels of $O_2^{\bullet -}$ were usually increased in plants grown in the presence of elevated concentrations of Fe, and it was especially evident in case of 'Amadeusz' (Table 2). 'Amadeusz' and 'Burundi' plants were characterized with almost unchanged levels of OH°, while in 'Omega' levels of OH° were strongly decreased. Activity of APX increased in examined cultivars, but it was least pronounced in the lowest Fe concentration. Fe affects CAT activity negatively in 'Amadeusz', but positively in 'Burundi' and 'Omega' (Table 2). Addition of Fe increased concentration of this element, but it was considerable only in the highest applied doses (Table 3). Concentrations of Mn and Zn was reduced and Ca unchanged in plants

grown in the presence of Fe. 'Amadeusz' and 'Burundi' were characterized with increased content of Mg, while 'Omega' decreased (Table 3).

Table 2. Levels of ROS and activity of anti-oxidative enzymes in leaves of lettuce and endive plants grown in nutrient solution enriched with Fe ions. Data are mean ±SE, n=3.

Cultivar	Measured parameter	Fe concentration (mg dm^{-3})			
		Control (12)	24	36	48
'Amadeusz'	anion-radical (relative values)	0.81	0.67	0.96	1.34
	hydroxyl radical (relative values)	0.12	0.12	0.12	n.m.
	ascorbate peroxidase (n kat g^{-1}FW)	7.85	10.78	10.98	16.67
	catalase (n kat g^{-1}FW)	0.24	0.16	0.14	0.21
'Burundi'	anion-radical (relative values)	0.45	0.63	0.48	0.48
	hydroxyl radical (relative values)	0.04	0.04	0.04	0.03
	ascorbate peroxidase (n kat g^{-1}FW)	4.97	5.35	9.21	7.47
	catalase (n kat g^{-1}FW)	0.30	0.33	0.26	0.36
'Omega'	anion-radical (relative values)	0.40	0.50	0.61	0.33
	hydroxyl radical (relative values)	0.16	0.12	0.07	0.07
	ascorbate peroxidase (n kat g^{-1}FW)	8.69	10.82	14.48	10.26
	catalase (n kat g^{-1}FW)	0.15	0.16	0.24	0.15

Table 3. Selected ions content in leaves of lettuce and endive plants grown in nutrient solution enriched with Fe ions. Data are mean ±SE, n=3.

Cultivar	Fe concentration (mg dm^{-3})	Examined element (mg g^{-1} DW)				
		Mg	Fe	Mn	Zn	Ca
'Amadeusz'	Control (12)	1.91	0.051	0.106	0.020	8.83
	24	1.76	0.051	0.067	0.013	7.91
	36	2.19	0.056	0.068	0.013	7.94
	48	2.44	0.101	0.040	0.013	9.27
'Burundi'	Control (12)	3.20	0.134	0.125	0.020	11.71
	24	3.18	0.143	0.101	0.019	11.78
	36	4.46	0.135	0.081	0.020	11.95
	48	3.67	0.157	0.073	0.018	12.29
'Omega'	Control (12)	2.78	0.068	0.132	0.022	9.98
	24	2.43	0.080	0.093	0.018	9.22
	36	2.46	0.083	0.080	0.019	10.14
	48	2.06	0.276	0.079	0.020	9.30

CONCLUSIONS

Addition of Fe do not influence strongly level of oxidative stress in examine leafy vegetables, but results of biofortification was not satisfactory, at least in this growing system.

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Variation in Amino Acid Composition and its relation to Mineral Nutrients in Grains of Wild Emmer Wheat Genotypes

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INTRODUCTION

Wild emmer wheat (*Triticum turgidum* ssp*. dicoccoides*) is proposed as an important genetic resource due to exceptionally high variation in grain Zn and protein content and compatibility to breeding-based biofortification strategies (Cakmak et al, 2004; Chatzav et al., 2010). Besides mineral element and protein content amino acid composition is also a key factor determining nutritional quality of cereal crops.

Cultivated wheat is usually low in micronutrients (i.e. Zn and Fe) and essential amino acids (i.e. Lys, Thr, Cys and Met) that are important to human nutrition (Cakmak et al, 2004; Acquistucci, et al., 1995) in the other hand it is rich in Glu and Pro. In wild emmer wheats, major (e.g. Glu and Pro) and minor amino acids (e.g. Met, Lys, His) can be 1.5 to 2 fold higher compared to cultivated wheats (Kabaha, 2010), hence exploitation of a certain trait (e.g. minor and/or essential amino acids) can simultaneously result in additional benefits such as high grain Zn and/or Fe. Therefore, this study aims to characterize the amino acid profile and its relation to mineral nutrients in grains of wild emmer wheat genotypes.

METHODS

Individual amino acids in whole meal flour samples of 14 wild emmer wheat genotypes were quantified by an amino acid analyzer (Biochrom 32 Oxidised Hydrolysate System, Biochrom Ltd., Cambridge, U.K.) following an accelerated protein hydrolysis method (Kabaha et al., 2011). Mineral nutrients except N were quantified by inductively coupled plasma optic emission spectroscopy (VistaPro Axial; Varian Pty Ltd, Mulgrave, Australia) following acid digestion. Nitrogen was quantified by an automated N analyzer (TruSpec CN, LECO Corp., Michigan, USA).

RESULTS AND DISCUSSION

The variation in concentration of individual amino acids increased in the order of Cys<Glu<Pro<Lys<His<Gly<Ser<Phe<Met<Ala<Asp<Val<Leu<Thr<Tyr<Ile<Arg (Table 1). In well agreement with literature knowledge, major wheat amino acids Glu and Pro were significantly higher (about two-fold) in wild emmer genotypes compared to cultivated wheats. However there was only a little variation in concentrations of major amino acids in wild emmer wheat genotypes tested here. Among the minor and essential amino acids Thr exhibited the highest variation (i.e. 0.251 to 0.504 g/100 g) followed by Met whereas others such as Lys and Cys were more or less stable (Table 1).

Among the mineral nutrients only N, S and Fe showed significant correlations with individual amino acids. Grain N concentration correlated with 9 out of 17 amino acids (Table 2). Since all amino acids comprise an amine (-NH_2) group and grain protein is composed of 16 % N it was not surprising to see such a correlation, particularly with Glu and Pro as well as with total amino acids. Other correlations were found between S and Leu, Cys, Asp and Thr and also between Fe and Thr, Leu, Tyr and Arg. However these correlations were not strong (i.e. $p< 0.05$) except for S and Leu ($p<0.01$). Correlation between Thr and Fe may be of particular importance for biofortification purposes as increasing grain Fe can at the same time influence Thr, an essential limiting amino acid in the wheat grain. However this point needs to be confirmed in other cereal species and environments.

Table 1. Amino acid concentrations in wild emmer wheat genotypes

Genotype	Cyst acid	Met sln	Asp	Thr	Ser	Glu	Pro	Gly	Ala	Val	Ile	Leu	Tyr	Phe	His	Lys	Arg	Total
									g/100 g wf									
TD 536	0.927	0.367	0.641	0.251	0.648	5.80	1.61	0.607	0.545	0.298	0.185	0.652	0.447	0.722	0.395	0.340	0.510	14.9
TD 531	0.869	0.363	0.783	0.395	0.876	6.98	2.04	0.665	0.607	0.616	0.475	1.142	0.627	1.046	0.416	0.379	0.546	18.8
TD 510	0.985	0.304	0.741	0.369	0.823	5.96	1.76	0.627	0.615	0.595	0.461	0.961	0.483	0.835	0.378	0.363	0.526	16.8
TTD 27	0.985	0.304	0.670	0.364	0.794	5.94	1.76	0.420	0.466	0.427	0.300	1.007	0.559	0.844	0.436	0.326	0.479	16.1
TD 195	0.870	0.255	0.862	0.475	0.887	6.08	2.18	0.696	0.832	0.598	0.532	1.312	0.728	0.967	0.419	0.392	0.736	18.8
TD 391	0.925	0.323	0.868	0.498	0.884	6.02	1.86	0.615	0.797	0.674	0.552	1.163	0.646	0.833	0.365	0.413	0.744	17.0
TTD 28	1.044	0.290	0.804	0.351	0.878	6.31	1.96	0.717	0.643	0.574	0.506	1.270	0.747	0.854	0.428	0.336	0.679	18.4
TD 390	0.989	0.300	0.829	0.470	0.901	6.37	2.09	0.667	0.696	0.615	0.565	1.349	0.695	0.970	0.423	0.407	0.903	19.2
TTD 89	0.903	0.287	0.892	0.487	1.003	6.86	2.32	0.790	0.904	0.641	0.574	1.415	0.841	1.051	0.457	0.376	1.099	20.9
TD 636	0.954	0.435	0.901	0.494	1.093	7.06	2.29	0.757	0.949	0.775	0.645	1.435	0.634	1.072	0.502	0.510	0.861	21.0
TTD 86	0.974	0.411	0.893	0.494	0.935	6.58	2.20	0.727	0.759	0.779	0.556	1.408	0.650	1.061	0.472	0.404	0.819	20.1
TTD 75	1.042	0.432	0.994	0.504	0.994	7.34	2.48	0.810	0.716	0.644	0.502	1.380	0.732	1.031	0.531	0.463	0.710	21.1
TD 399	1.004	0.434	0.670	0.293	0.548	5.80	1.89	0.631	0.861	0.712	0.536	1.119	0.372	0.682	0.364	0.472	0.662	17.0
TTD 18	0.876	0.266	0.388	0.305	0.739	6.25	1.78	0.514	0.675	0.674	0.557	0.772	0.386	0.544	0.269	0.316	0.523	15.8
mean	0.953	0.341	0.781	0.411	0.857	6.38	2.01	0.660	0.719	0.616	0.496	1.170	0.611	0.894	0.418	0.393	0.700	18.3
stdev	0.060	0.065	0.152	0.089	0.143	0.50	0.25	0.105	0.140	0.127	0.119	0.247	0.143	0.163	0.065	0.058	0.179	2.0
cv (%)	6	19	20	22	17	8	13	16	19	21	24	21	23	18	16	15	26	11

Table 2. Correlations between amino acids and mineral nutrients in wild emmer wheat genotypes.

	N	K	P	Mg	S	Ca	Fe	Mn	Cu	Zn
Cys	0.401	-0.004	-0.131	0.433	0.611*	0.062	0.101	0.213	0.361	0.165
Met	0.472	0.099	0.221	-0.024	0.114	-0.081	-0.202	-0.288	-0.208	-0.526
Asp	0.675**	0.068	0.046	0.479	0.595*	-0.011	0.506	0.116	0.383	0.12
Thr	0.52	0.072	-0.127	0.308	0.574*	-0.105	0.654*	-0.022	0.39	0.198
Ser	0.646*	-0.108	-0.087	0.134	0.402	-0.287	0.342	-0.096	0.434	0.173
Glu	0.871***	0.033	-0.01	0.047	0.17	-0.31	0.003	-0.273	0.364	-0.122
Pro	0.816***	0.337	-0.02	0.348	0.483	0.032	0.333	-0.255	0.345	0.198
Gly	0.711**	0.156	0.058	0.246	0.291	-0.142	0.13	-0.103	0.168	-0.031
Ala	0.243	0.434	0.114	0.071	0.199	-0.097	0.298	-0.119	-0.324	0.079
Val	0.354	0.148	-0.03	-0.077	0.364	-0.354	0.265	-0.068	-0.113	-0.064
Ile	0.328	0.219	-0.174	0.068	0.365	-0.338	0.397	0.03	-0.048	0.101
Leu	0.696**	0.196	-0.13	0.473	0.687**	-0.046	0.597*	0.092	0.242	0.333
Tyr	0.626*	0.021	-0.093	0.508	0.522	-0.023	0.559*	0.158	0.482	0.473
Phe	0.692**	-0.099	-0.075	0.253	0.397	-0.109	0.367	0.037	0.323	0.076
His	0.805***	0.066	0.059	0.391	0.505	0.117	0.209	-0.145	0.394	0.154
Lys	0.451	0.421	0.055	0.24	0.327	-0.023	0.24	-0.151	-0.147	-0.281
Arg	0.385	0.091	-0.283	0.236	0.337	-0.192	0.540*	0.125	-0.031	0.313
Total	0.815***	0.155	-0.093	0.276	0.462	-0.135	0.325	-0.111	0.281	0.152

*,** and *** indicate significance at P≤0.05, 0.01, 0.001 respectively

CONCLUSIONS

There exists a substantial genetic variation among wild emmer wheat genotypes which can be exploited to increase specific and/or total amino acids (i.e. protein) in high yielding cultivated wheats through genetic biofortification. Among correlations between mineral nutrients and amino acids, N expressed highest number of significant associations with a majority of amino acids which may be attributed to protein-N in general. There was no convincing evidence of a co-transport or co-accumulation of a specific amino acid with a specific mineral nutrient. Future research may focus on phloem transport and mobility of metal binding proteins and organic ligands rather than individual amino acids to characterize grain deposition of mineral nutrients.

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