# Effect of elemental sulphur and sulphate additions to soil on zinc biofortification in wheat grains

**Master Thesis**

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**Publication date:**
2011

**Permanent link:**
https://doi.org/10.3929/ethz-a-006708459

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Institute of Terrestrial Ecosystems

# EFFECT OF ELEMENTAL SULPHUR AND SULPHATE ADDITIONS TO SOIL ON ZINC BIOFORTIFICATION IN WHEAT GRAINS

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**Master Thesis**

*Zurich, September 2011*

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Picture on the front page: Wheat (Flora Conte)

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**Aknowledgements**

In the first place I would like to thank the people of the Soil Protection Group for the wonderful time I had the chance to spend with you. You are interested, interesting, motivated, friendly, open, efficient, serious when it is necessary and very funny the rest of the time. I will definitely miss you.

I would like to thank Prof. Rainer Schulin for having introduced me to the research area situated between environmental protection, agriculture and development that fascinates me so much, during this Master Thesis and during my studies in general.

Special thanks go to Dr. Susan Tandy, who was a perfect supervisor during these months. Although I was expected to be nervous, I felt serene most of the time thanks to your help and advices.

All my gratitude to Björn Studer, René Saladin and Dr. Michael Evangelou who helped me in the lab and made me lose my fear of machines and chemicals. I finally started to like lab work! Thank you to Gilbert Gradinger for the technical computer support - the new screen made life so much easier! – and to Dr. Andreas Papritz for the statistical advices.

I would also like to mention all the people I met this year at the Blinkmatt farm as well as in Niger. They allowed me to aerate my head and to make the link between the topic of my thesis and reality. I discovered crops, alkaline-sandy soils, organic and mineral fertilisers, air pollution and fresh air, and how children can have too much energy or not enough micronutrients.

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## Abstract

Zinc (Zn) deficiency is a major problem for human health in regions where diets consist mainly of staple-foods. This lack of micronutrient may be lowered by Zn biofortification of wheat grains. Sulphur's (S) effect on Zn concentrations has been studied less than the influence of nitrogen (N), although both are main constituents of proteins. New fertilisers and air pollution reduction have resulted in S deficiency in many agricultural soils. Elemental sulphur ($S^0$) may be used to acidify the soil while oxidising to sulphate ($SO_4$), and thus to increase the bioavailability of trace elements such as $Zn^{2+}$. The aim of this study was to compare the interactions of Zn and S while differentiating between $S^0$ and $SO_4$ additions, in order to determine whether the metabolic function of S or the pH effect and the slow-release capacity of $S^0$ were responsible for previously reported increases in Zn grain concentrations. Spring wheat (*Triticum aestivum*) of the variety Kavir was grown in pots filled with a Zn- and S deficient, alkaline, and sandy substrate in a climate chamber under controlled conditions. After basal fertilisation six treatments were compared: Zn, $S^0$, $SO_4$, Zn+ $S^0$, Zn+ $SO_4$, and a control. Zn additions were 2 mg $kg^{-1}$of Zn-$ZnCl_2$ and 100 mg S $kg^{-1}$ was added as $S^0$ or $K_2SO_4$. One pot per treatment was left unplanted to observe pH, available Zn and available $SO_4$ changes with time. Wheat plants were harvested at maturity. DTPA-extractable Zn and $KH_2PO_4$ extractable $SO_4$ were measured in soils and Zn, S, P, and other elements were analysed in grains and shoots after harvest with ICP-OES. Soil pH slightly increased, contrary to our expectations, and no Zn was mobilised from the soil. However, higher available manganese (Mn) concentrations in soils indicated that a pH decrease took place locally. $SO_4$ release from the soil slowly increased with time when $S^0$ was added. Zn and S total contents and concentrations respectively were positively correlated in grains, indicating a link between both elements in protein metabolism and storage in grains. $S^0$ and $SO_4$ had significantly different influence on total Zn and S contents in grain, but not on grains Zn and S concentrations. However, clear differences between $S^0$ and $SO_4$ treatments were observed during plant growth and in grain and shoot yields. The slow-release of $SO_4$ by $S^0$ allowed wheat to reach maturity faster and thus to remobilise Zn from leaves to grains. In $SO_4$ treatments, although total shoot S content was higher than for $S^0$ and Zn+$S^0$, available soil $SO_4$ was too low during grain-filling and could not be remobilised from its high shoot biomass. Differences between $S^0$ and $SO_4$ were thus mostly due to changes in availability on soils with time. The use of $S^0$ showed clear advantages over $SO_4$ if used in alkaline soils.

## Zusammenfassung

In Gegenden, in denen man sich hauptsächlich mit Grundnahrungsmitteln ernährt, ist Zinkmangel ein besonders ausgeprägtes Problem für die menschliche Gesundheit. Biofortifikation ist ein möglicher Ansatz, um den Gehalt dieses Spurenelementes in Weizenkörnern zu erhöhen. Der Einfluss von Schwefel (S) auf den Zinkgehalt wurde bis heute weniger erforscht als der von Stickstoff (N), obwohl N und S Haupbestandteile der Proteine sind. Wegen neuer Dünger und abnehmender Luftverschmutzung tritt S-Mangel in Böden vermehrt auf. Durch die Oxidation von elementarem Schwefel ($S^0$) zu Sulphat ($SO_4$) werden Böden angesäuert, um die Verfügbarkeit von Spurenelementen wie Zn zu erhöhen. Ziel dieser Studie war es, Zn-S Interaktionen mit Hinblick auf den Unterschied zwischen $S^0$ und $SO_4$ zu vergleichen, d.h. die Frage zu stellen, ob die metabolische Funktion von S oder der pH Effekt und die langsame Abgabe von $S^0$ den Zn-Gehalt in Körnern beeinflusst. Sommerweizen (*Triticum aestivum*) der Sorte Kavir wurde während eines Topfexperimentes in einer Klimakammer in einem Zn- und S-armen, sandigen, alkalischen Boden gezüchtet. Nach Grunddüngung des Bodens wurden sechs Behandlungen verglichen: Zn, $S^0$, $SO_4$, Zn+ $S^0$, Zn+ $SO_4$, und eine Kontrolle. 2 mg $ZnCl_2$-Zn $kg^{-1}$ und 100 mg S $kg^{-1}$ wurden als $S^0$ oder $K_2SO_4$ beigegeben. Um die Veränderung des pH, verüfgbaren Zn und S mit der Zeit beobachten zu können wurde ein Topf pro Behandlung nicht bepflanzt. Der Weizen wurde im reifen Zustand geerntet. Der Bodengehalt an DTPA-extrahierbaren Zn und $KH_2PO_4$-extrahierbaren $SO_4$, sowie Zn, S, P und andere Elemente in Weizenkörnern und –blättern wurden mit ICP-OES analysiert. Entgegen der Erwartungen stieg der Boden pH leicht an und kein Zn wurde vom Boden mobilisiert. Der Anstieg des verfügbaren Mangans (Mn) im Boden wies jedoch darauf hin, dass der pH lokal sank. $S^0$ Einträge bewirkten eine langsame $SO_4$ Abgabe an den Boden. Zn und S Totalgehalte pro Pflanze sowie Konzentrationen waren in Körnern positiv korreliert, was ein Zusammenhang beider Elemente in Proteinmetabolismus und –speicherung andeutete. $S^0$ und $SO_4$ wirkten unterschiedlich auf den Zn- und S-Gehalt pro Pflanze in Körnern, aber nicht auf Zn- und S Konzentrationen. Dennoch wurden deutliche Unterschiede zwischen $S^0$ und $SO_4$ während des Pflanzenwachstums und in den Korn- und Blatterträgen beobachtet. Durch die langsame $SO_4$-Abgabe durch $S^0$ konnte der Weizen schneller reifen und so Zn von den Blättern in die Körner remobilisieren. In den $SO_4$ Behandlungen war das verfügbare $SO_4$ während der Kornfüllung zu niedrig und konnte nicht von der hohen Blattbiomasse remobilisiert werden. Die Unterschiede zwischen $S^0$ und $SO_4$ waren grösstensteils wegen der Veränderungen der Verfügbarkeit im Boden im Laufe der Zeit zu erklären. Der Gebrauch von $S^0$ in alkalischen Böden zeigte klare Vorteile gegenüber $SO_4$.

# 1 Introduction

Wheat is one of the most important cereals grown in the world. In 2011/2012 the global production of wheat is forecasted to be 674 million tons (FAO, 2011). It is grown, produced and consumed in developing as well as in industrialised countries. Since it is one of the oldest domesticated cereals, a very wide range of wheat genotypes exist all over the world, allowing wheat to be grown under very different temperature and moisture conditions. For example, the optimal temperature range for germination varies between 12 and 25 °C (Acevedo et al., 2002).

While the world population is increasing exponentially and not only water, but also agricultural land is becoming a scarce resource, wheat yields have to be constantly increased in order to meet the higher demand. Wheat seeds contain 120 g $kg^{-1}$ protein (Egli, 1998), which is more than the other major food crops maize (100 g protein $kg^{-1}$) and rice (80 g protein $kg^{-1}$). It is used in a wide variety of different foods such as bread, pasta, and pastry.

A diet mainly consisting of wheat products is not sufficient to meet nutritional health requirements. Bioavailable essential micronutrients such as iron and zinc are mostly present in non-staple foods, particularly dairy and meat products, but also fruits, vegetables, or pulses. However these products are less affordable than staple-foods. The poorer the populations the more they rely on consumption of cereals such as rice and wheat, which are often their primary sources of micronutrients (Bouis, 2003). Unfortunately in the last decade, except during the year 2008, global non-staple food prices have been constantly rising and cereals prices increased even more (FAO, 2011, Figure 1). The food price evolution as well as water scarcity may induce a more vegetarian diet, enhancing wheat consumption and increasing the risk of micronutrient deficiencies. So, although specific foods containing relatively high concentrations of Zn can be found in most parts of the world, the consumption of food containing low Zn contents such as wheat products is constantly rising.

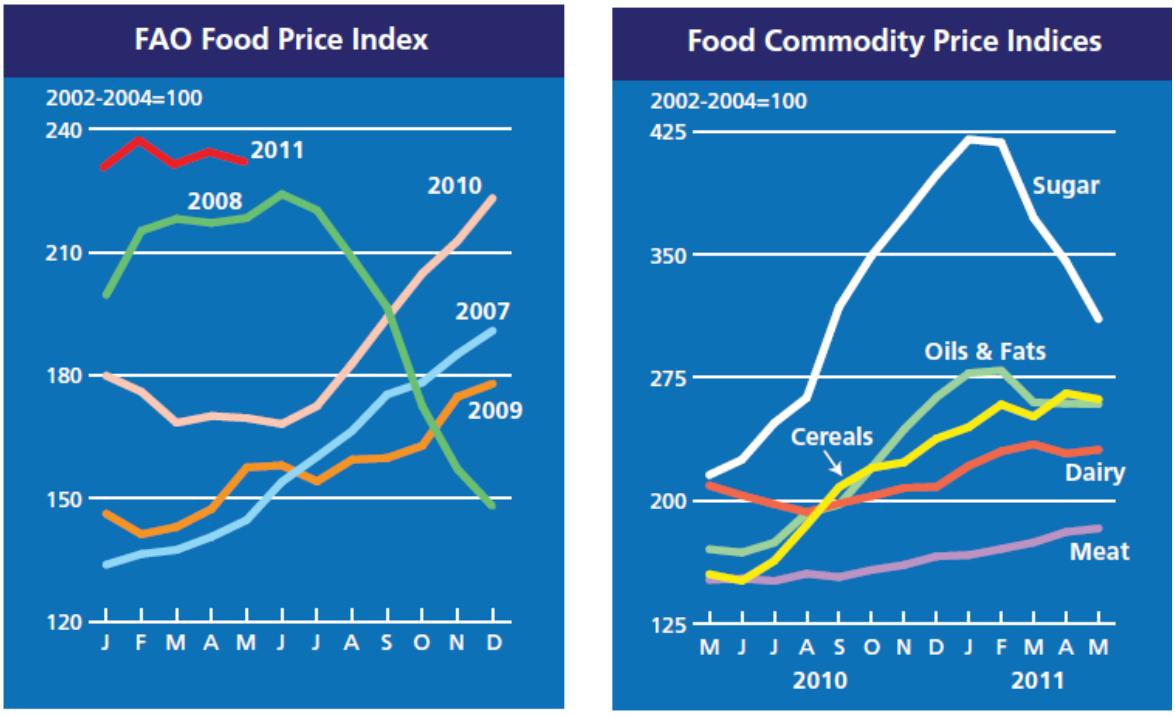



**Figure 1:** FAO Food Price Index (left) and FAO Food Commodity Price Indices (right) *(FAO, 2011)*

According to the World Health Report (WHO, 2002), micronutrient deficiencies represent major health risks. Zinc, iron, vitamin A and iodine are the most prevalent nutrient deficiencies. They affect a larger part of the world's population than does protein-energy malnutrition and also concern plant and animal health. Women and children are more affected by micronutrient deficiency than men (Bouis, 2003). Zinc deficiency touches one third of the world population. Severe zinc deficiency is much less frequent than a century ago. However worldwide, it causes 0.8 million deaths. It has been observed to induce short stature, disorders such as impaired immune function. It is responsible for approximately 16% of lower respiratory tract infections, 18% of malaria and 10% of diarrhoeal disease, which, in turn, also causes excessive zinc losses (WHO, 2002). These health problems are known to be major reasons for child mortality (Fischer Walker et al., 2009, Figure 2).


**Figure 2:** IZA sensibilisation campaign on children zinc deficiency

One reason why zinc deficiency has such important impacts on humans is that it has a major role in many aspects of the immune system. Zinc deficiency negatively affects cells mediating innate immunity, i.e. NK cells, neutrophils, macrophages, phagocytosis, cytokine production, T and B cell growth. In addition Zn acts as an anti-oxidant and stabilizes membranes. Numerous enzymes and transcription factors also contain zinc (Prasad, 2008). In plants and animals, zinc is at least as important as in humans.

There are different ways to counter nutrient element deficiencies:

- direct additions for humans and animals: dietary supplementation (tablet form), zinc addition to the final product, or fertilizers for plants
- biofortification, i.e. increasing the zinc concentration in crops themselves. Biofortification can be achieved by selective breeding by either conventional means or genetic modification or by agricultural practises such as fertilisation.

Biofortification has several possible drawbacks. Plants may take up other non-essential elements as well or instead of the desired trace elements. If the zinc is added to the soil in deficient areas, care must been taken to avoid application that may lead to Zn toxicity (Robinson et al., 2009). One alternative could be seed coating (Alloway, 2009; Central Valley Coop, 2011). Farmers have to accept the use of new crop varieties in order to implement genetic biofortification. In the case of genetically engineered crops, issues such as patent protections and public acceptance have to be considered. However biofortification has many complementary advantages compared to direct zinc addition to food products: (i) lower risk of toxicity if Zn is directly taken up from the soil pool instead of adding high amounts of fertilisers, (ii) physiologically accumulated Zn in plants often has a higher bioavailability to humans than food supplements, (iii) livestock can simultaneously benefit from it (Robinson et al., 2009), (iv) no behavioural change for consumers, (v) reaching people in isolated, rural areas, since food production can take place on the site and does not imply commercial activities, (vi) crops often attain higher yields due to sufficient micronutrient supply and may be more resistant to diseases, (vii) micronutrient-efficient varieties usually have a more developed root system, which may reduce the expenses for fertilisers and irrigation (Bouis, 2003).

The goal of biofortification to improve human health could, thus, also help enhance yields by treating the problem of zinc deficiency in plants. However, according to Liebig's law of the minimum, the deficiency of a micronutrient such as Zn is only relevant for the plant if no other nutrients are limiting. Healthy plant growth also requires sufficient available quantities of nitrogen, phosphate, potassium, sulphur and other nutrients. Despite intensive agronomic research on macronutrient deficiencies in major crops, not all processes are understood since agricultural practices and the balance between natural and anthropogenic inputs of those nutrients is constantly changing. Sulphur inputs to plant and soil can be very heterogeneous over time and space, due to factors such as air pollution – sulphur dioxide emissions have decreased since the 1970's (Zhao and McGrath, 1994) - or changing nitrogen fertilisation practices – the use of ammonium sulphate is decreasing (Cui and Wang, 2005).

It is important to look at the interactions between sulphur and zinc in plant development, especially in the context of wheat biofortification. On the one hand, the biochemical sulphur cycle in plants is tightly linked to the zinc cycle because both elements are major constituents of essential proteins and play important roles in metabolic processes such as photosynthesis (Broadley et al., 2007; Bergmann, 1992; Cakmak, 2000; Zörb et al., 2010). On the other hand, sulphur oxidation to sulphate compounds generates acidity (Formula 1). The availability of metal cations such as $Zn^{2+}$ is clearly correlated to soil pH. The solubility and phytoavailability of $Zn^{2+}$ decrease as soil pH increases (Marschner, 1986). Acidification means more positively charged soil particles, a change in metal speciation, and a shift in the oxidation-reduction reactions of metals in the soil (Scheffer and Schachtschabel, 2002). Elemental sulphur in soils needs to be oxidized to sulphate for plant uptake. This oxidation is microbially mediated. Whereas elemental sulphur additions imply a slow release - with a rate depending on climatic, biological and chemical factors, directly adding sulphate to the soil allows sulphur to be immediately available for plants. So sulphur may have two effects on zinc uptake from the soil: acidification by elemental sulphur oxidation and sulphur's essential role as a macronutrient. These effects should be differentiated in order to better understand how wheat grains can contain more zinc and, thus, reduce Zn deficiency in humans.

$$2S^0 + 2H_2O + 3O_2 \rightarrow 2SO_4^{2-} + 4H^+$$

**Formula 1:** Oxidation of elemental sulphur

Although research has focussed more on nitrogen, phosphorus, and potassium, sulphur is known to be an essential macronutrient for plants. The influence of elemental sulphur on zinc uptake has been analysed in plants (Cui and Wang, 2005; Togay et al., 2008, Fässler et al., 2010), and in wheat in particular (Singh et al., 1986). However the potentially enhanced zinc uptake due to acidification resulting from $S^0$ oxidation was not sufficiently studied while taking into account sulphur's importance for essential plant functions, maybe because the extent of sulphur deficiency has been underestimated in the last decades. When nitrogen supply positively affects Zn concentrations (Erenoglu et al., 2011), this is automatically linked to protein metabolism because N is a major constituent of proteins, whereas sulphur, which is also contained in protein has not been given as much attention from research on Zn grain concentrations. Kutman et al. (2010) mention that there is a lack of knowledge about the physiological factors affecting root uptake, root-to-shoot transport, phloem loading, remobilisation of Zn from source tissues into developing seeds and seed deposition of Zn. Fertilisation with $SO_4^{2-}$ or $S^0$ may influence the duration of growth stages and the time needed by wheat to reach maturity and it is not clear whether zinc accumulation in wheat grains is more influenced by leaf senescence or by changes in the grain-filling period (Jang and Zhang, 2006).

In order to get a better knowledge on potential methods of biofortification, the role of sulphur on zinc uptake in wheat was investigated in this master thesis. Elemental sulphur and sulphate additions will be studied separately in order to try to answer following questions:

- Does the available soil sulphate concentration influence zinc uptake in wheat grains?
- Does the form of added sulphur fertiliser influence S and Zn contents in wheat grains differently?
- Is the pH effect of $S^0$ oxidation or sulphur's role as a macronutrient responsible for the results of previous studies showing how S inputs enhanced zinc uptake in wheat?
- Is there a link between zinc uptake of wheat grains and sulphur additions that is similar to the better studied relation between zinc uptake and nitrogen fertilisation?
- Can Zn uptake by the grains still be enhanced if wheat biomass increases?
- Does leaf senescence stimulate the Zn transport into seeds or does rather delayed senescence increase grain Zn accumulation due to a longer grain-filling phase?

**Hypothesis:**

We will try to answer the research questions listed above by verifying the following hypothesis:

1. Zn and S fertilisation have a synergistic effect on grain Zn concentration because S and Zn are associated in grain proteins.

2. Elemental sulphur additions have more effect on wheat grain Zn concentrations than sulphate because it increases zinc solubility in soils by lowering pH.

3. Elemental sulphur additions enhance wheat grain yields more than sulphate because the sulphate release in the soil solution is slower so that there is still sufficient available sulphate during the grain-filling phase.

4. Due to S deficiency during generative growth stages, wheat maturity is reached later with sulphate additions than with elemental sulphur inputs, so that remobilisation of Zn from the vegetative tissue to the grains is hindered.

# 2 Theoretical Background

## 2.1 Wheat

Wheat is one of the three most important cereals in the world and one of the earliest food crops domesticated. It was first planted around 8000 BC in the Fertile Crescent. Today, there is a very wide range of wheat species and varieties. This explains why wheat can adapt to the most different environments. It grows at altitudes between sea level and 3500m and latitudes between 60° North and South (except in lowland tropics). Wheat height also varies between 30 and 120 cm. Since wheat plants produce many tillers, seeds can be sown sparsely. Seed size varies between 20'000 and 53'000 seeds per kg. The optimum growth temperature is 25-27°, but wheat can grow in the range of 4 to 40°. The growth period takes between 95 and 150 days. Yields vary substantially in different regions of the world, from 300 kg/ha in the poorest regions to more than 10 tons/ha with optimal conditions and varieties. Parameters influencing wheat yields include disease, the tendency to be flattened during storms, soil drainage, and fertiliser use. Wheat is mostly grown for its grains, and the amount and quality of gluten in wheat grains is an important factor for flour production (Winch, 2006).

The classification of *Triticum aestivum* is shown in Table 1. Since the tribe Triticeae includes around 400 species, there is a large gene pool that could allow genetic wheat improvement e.g. resistance genes, dwarfing genes (Chopra and Prakash, 2002). This is partly due to the fact that wheat species are polyploid. They have between 2 and 6 sets of chromosomes. *Triticum aestivum* is allohexaploid.

**Table 1:** Taxonomy of bread wheat

| **Classification of *Triticum aestivum*** | |
|---|---|
| Plant kingdom | Angiosperm (monocot) |
| Order | Poales |
| Family | Poaceae |
| Tribe | Triticeae |

The most important growth stages according to the Feekes scale are shown in Figure 3. The detailed order of Feekes stages and other development scales for cereals are listed in the Appendix. During growth, after germination, the wheat tillers quickly, i.e. develops more than one shoot. The first tiller appears after the emergence of the fourth leaf. According to Satorre and Slafer (1999), if resources are unlimited, tillers appear following the scheme of a Fibonacci series, i.e. each subsequent number of tillers is the sum of the previous two. However when resources become limiting, tiller appearance slows down and some tillers die, in the reverse order of appearance. Generally, tiller mortality happens at the same time as stem elongation. The tillering period stops when the initiation of the terminal spikelet on the main shoot apex starts (Satorre and Slafer, 1999). During stem elongation, nodes develop on the stems. The spike grows inside the last leaf, called flag leaf, and is constituted of spikelets (Figure 4). Before emergence, the spike forms a “boot” inside the flag leaf. As soon as this boot is opened, the heading period begins. After anthesis (flowering), the spike ripenes, and the whole plant dries. The words “head” or “ear” are synonyms for “spike”, and “seeds” and “kernels” are synonyms for “grains”.

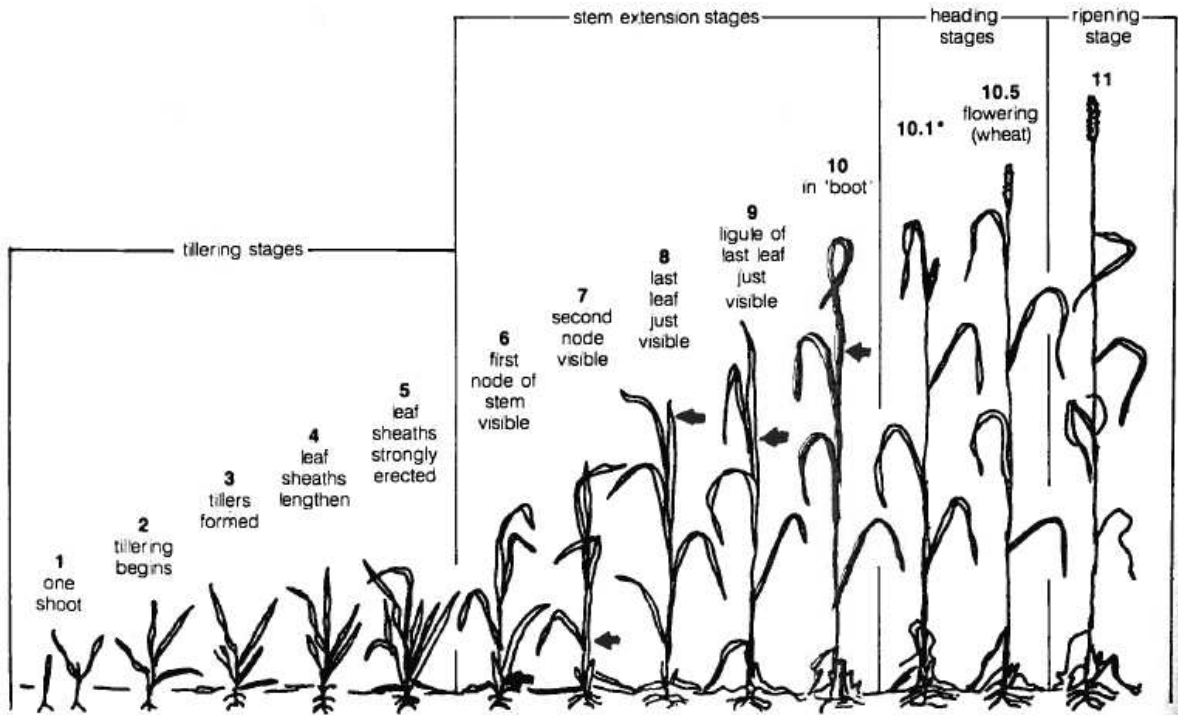



**Figure 3:** Wheat growth, Feekes stages *(Reuter and Robinson, 1986)*

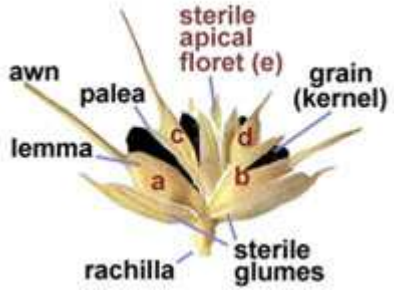



**Figure 4:** Central spikelet of a wheat spike *(FAO)*

The anatomy of a wheat seed is shown in Figure 5. The endosperm, consisting of dead cells packed with starch and some protein, is surrounded by the aleurone layer. It can represent 80% of the dry weight of a mature seed. Assimilates are unloaded from the phloem into a single vascular bundle embedded in the maternal tissue (aleurone). They must move apoplastically into the embryo and the endosperm before being taken up by the cells (Egli, 1998). Storage protein represents 2/3 of the total protein content in wheat grains (Zörb et al., 2010). Several molecules of nutritional interest such as vitamins and minerals are stored in the aleurone cells, which contain 46% w/w proteins and 40% w/w phytic acids.

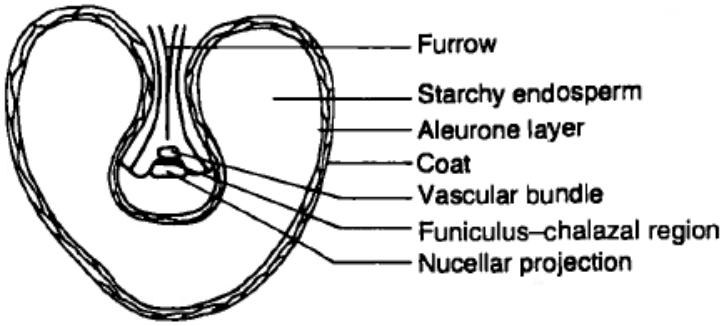



**Figure 5:** Cross-section through a developing wheat seed *(Egli, 1998)*

The most important period for protein and starch content of seeds is the period after fertilisation. Egli (1998) divides the development of seeds between fertilisation and maturity into three phases:

- Phase I: fertilisation and rapid cell division. All the structures are formed during this phase.
- Phase II: Seeds accumulate reserve materials at a constant, maximum growth rate.
- In phase III, the material accumulation slows down and water concentration reaches a minimum until physiological maturity is reached.

Physiological maturity is an important growth stage. At this stage, the seed has reached maximum dry weight and the end of the seed filling period. It is thus the end of active plant growth and yield production. Yields are only affected by plant and environmental factors such as precipitation before physiological maturity. Not all seeds will reach this stage at the same time, but absolute physiological maturity is not essential for most practical applications. Changes in seed colour, seed structure or the spike are used to determine if this stage has been reached. For instance, loss of green colour from most of the portions of the spike is a good indicator for physiological maturity of wheat. Seeds from late-developing flowers frequently have shorter developmental periods. This could affect their composition. The relationship between seed fill duration and seed composition is not well known for most crop species (Egli, 1998).

Spring wheat (*Triticum aestivum*) of the Iranian cultivar *Kavir* was used for this experiment. It is a rather salinity tolerant and zinc inefficient variety (Koshgoftar et al., 2006; Koshgoftarmanesh et al., 2009).

## 2.2 Zinc in soils

In nature plants obtain Zn and other nutrients from the soil. The global mean total zinc content in soils is estimated to be 64 mg Zn $kg^{-1}$, but variations even within one country, can be of three orders of magnitude, e.g. in England and Wales, the total soil zinc range is 5 to 3548 mg $kg^{-1}$ (Alloway, 2009). The common range for total Zn in soils is 10 - 300 mg $kg^{-1}$. Total soil Zn content is largely dependent on the composition of the parent rock material. Zinc is present in minerals due to non-specific replacement of iron and magnesium. The most abundant sources of Zn in the lithosphere are the ZnS minerals sphalerite and wurtzite, with mean lithospheric zinc contents of 40 mg $kg^{-1}$ in acid rocks (granites) to 100 mg $kg^{-1}$ in basaltic rocks. Clayey sediments have the highest Zn content among sediments (80-120 mg $kg^{-1}$), while sandstone rocks only contain 10-30 mg Zn $kg^{-1}$.

Zn is also deposited to the soil from the atmosphere. Natural aerial sources are volcanic eruptions and aeolian dust (Kiekens, 1995). However anthropogenic Zn sources are more than 20 times higher than natural emissions (Broadley et al., 2007). Burning of coal and other fossil fuels, and the smelting of non-ferrous metals generate Zn inputs. Air Zn concentrations largely vary between very remote areas and industrialised countries (0.002-0.05 ng $m^{-3}$ in the South Pole to 14 to 6800 ng $m^{-3}$ in Japan). Sewage sludge contains appreciable amounts of Zn denpending on its source. Fertilisers - mineral as well as organic, soil amendments such as limestone and manure, and pesticides also contain Zn impurities that increase Zn concentrations in soils (Kiekens, 1995).

*Chemical behaviour of Zn in soils*

Kiekens (1995) divides Zn in soils into the following more or less distinct fractions:

a) Water-soluble pool: fraction present in the soil solution and bioavailable.
b) Exchangeable pool: ions bound to soil particles by electrical charges.
c) Adsorbed, chelated or complexed pool: metals bound to organic ligands
d) Pool of clayey secondary minerals and insoluble metallic oxides.
e) Pool of primary minerals.

The parameters determining interactions between Zn fractions in a system are:

a) Concentrations of $Zn^{2+}$ and other ions in the soil solution
b) Type and amount of adsorption sites associated with the solid phase of the soil
c) Concentrations of all ligands capable of forming organo-zinc complexes
d) pH and redox potential of the soil (Kiekens, 1995).

More than 90% of soil zinc is insoluble and thus unavailable for plants (Broadley et al., 2007). According to Kiekens (1995), concentrations of Zn in soil solution range from $3\ 10^{-8}$ and $3\ 10^{-6}$ M. The equilibrium of $Zn^{2+}$ activity is displaced as a result of plant uptake, losses by leaching, inputs of Zinc by different routes, changing moisture content of the soil, pH changes, mineralisation of organic matter and changing redox status of the soil. The activity of $Zn^{2+}$ in the soil solution is directly proportional to the square of the proton activity. Below pH 7.7, $Zn^{2+}$ seems to be the predominant species, while $ZnOH^{+}$ and later $Zn(OH)_2$ are predominant above this pH. Zn may also be present in the soil solution as organic species. The most important components contributing to adsorption of Zn are clay minerals, hydrated

metal oxides and organic matter, i.e. the colloidal phase of the soil, which has usually a negative charge compensated by equivalent amounts of positive charges (Kiekens, 1995).

*Zinc deficiency*

The combination of certain soil characteristics induces Zn deficiency for plants. Either the total Zn concentration is low, or the bioavailable Zn fraction is not sufficiently high. DTPA-TEA extractable soil zinc concentrations between 0.5 and 1.5 mg $kg^{-1}$ dry soil are typically critical for plant growth (Alloway, 2009). Plants take up zinc mainly as $Zn^{2+}$. However this cation has a relatively low mobility in soils (Bergmann, 1992, Jones and Jacobsen, 2001). Thus the uptake has to take place mainly at the direct root-soil interface (rhizosphere), which is difficult to determine satisfactorily (Broadley et al., 2007).

India, China, Pakistan, Turkey, Iran, and Bangladesh are the countries with the highest estimated proportion of zinc deficient soils (Alloway, 2009). In addition soils of many other regions are zinc deficient, which are mostly located in developing countries, for instance Brazil, many African countries and Mexico (Figure 6).

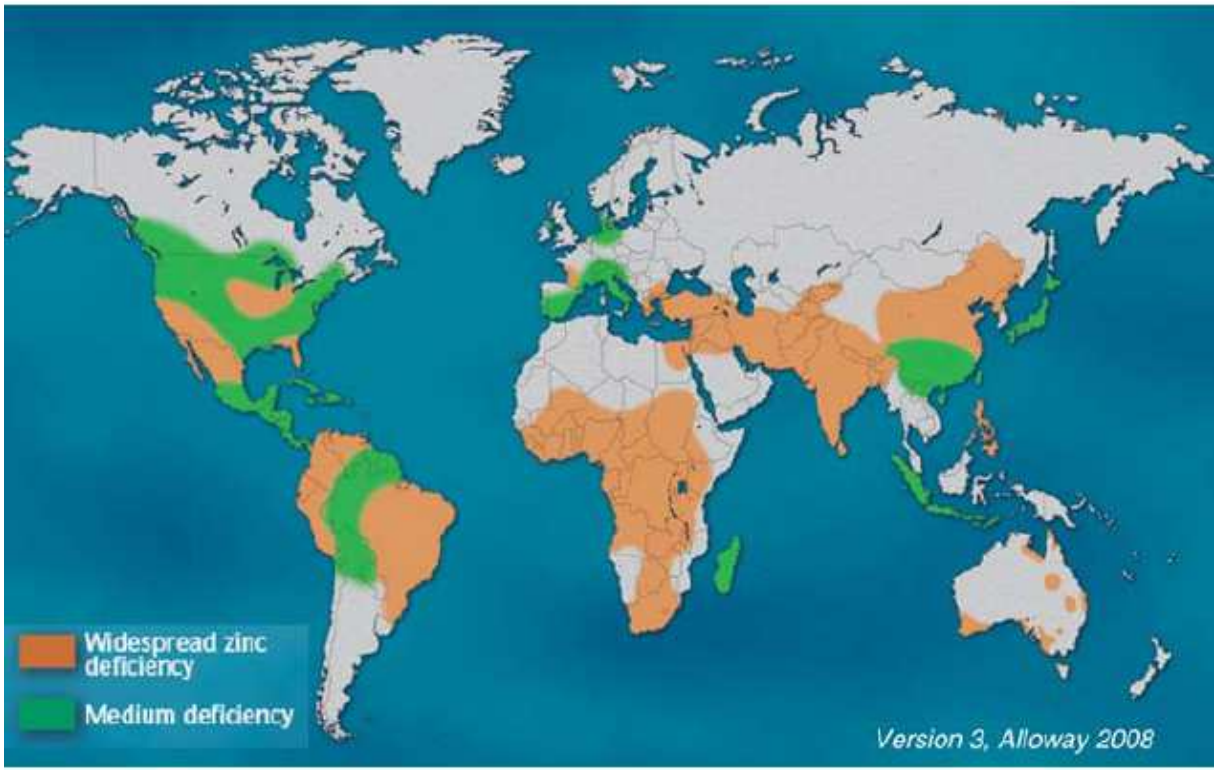



**Figure 6:** Global distribution of zinc deficient soils

Different studies on zinc deficiencies in soils describe numerous conditions potentially leading to zinc deficiency (Bermann, 1992; Follet and Westfall, 2004; Alloway, 2008 and 2009). The main soil parameters influencing Zinc uptake of plants are pH, phosphate concentration, organic matter content, water content, soil texture, and sorption capacity. This is why Zn deficiency is expected in:

- Acid and highly weathered soils (tropical soils) due to a low total Zn content caused by a lack of adsorption and leaching.
- Soils with neutral to alkaline pH, particularly calcareous soils because of low available Zn concentrations.
- Soils with a high phosphate content due to more negatively charged sites leading to Zn adsorption, as well as reduced infection by mycorrhiza, which take up Zn.
- Soils with high salt concentrations (Na, Ca, Mg, bicarbonate etc.) due to cation competition.
- Soils with a very high organic matter content, which adsorbs Zn.
- Coarse/sandy soils or subsoils due to poor nutrient retention on clay or iron oxides.
- Waterlogged soils. Cold and wet conditions provoke soil compaction and inhibit microbially mediated zinc release.

Ideal soil conditions for a sufficient Zn nutritional status include a pH, water content, clay and organic matter content which is neither too low nor too high.

*Countering soil zinc deficiency*

There are two commonly used solutions to correct zinc deficiency in soils: (i) addition of zinc fertilisers to the soil or as foliar spray, or (ii) acidification of the soil in order to increase zinc availability.

The most commonly used inorganic zinc fertilisers are zinc sulphate ($ZnSO_4*7H_2O$, with 26% Zn, $ZnSO_4*H_2O$, 37% Zn), zinc chloride ($ZnCl_2$), zinc nitrate ($Zn(NO_3)_2$), zinc oxide (ZnO) or Zn coated urea. Superphosphate contains Zn impurities. The application rates usually vary between 4.5 and 34 kg Zn $ha^{-1}$. Zn may be added as foliar spray in chelated forms such as $Na_2Zn$ EDTA (Alloway, 2009). Organic zinc fertilisers can be chelates or manure if the applied amounts are sufficiently high (Follet and Westfall, 2004).

Soil zinc bound to soil surfaces such as organic matter particles, oxides, and clays can be made more available by lowering the pH. Zinc deficiency is common if the pH is higher than 7. For instance, the acidification from pH 6.5 to 5.3 may increase zinc availability by up to 50% (Bergmann, 1992). The addition of mineral or organic acids or acid-producing fertilisers is a way to decrease soil pH (Cui and Wang, 2005).

### 2.3 Zinc in wheat

Zinc has an oxidation state of +2 and has a small radius to charge ratio. This means it forms strong covalent bonds with S, N and O donors and, thus, many soluble salts. Zinc is an essential element, not only in humans and animals, but also in plants. After iron, zinc is the most abundant transition metal in organisms. It is present in all six enzyme classes (Broadley et al., 2007) and is needed for the catalytic function and the structural integrity of proteins more than any other metal (Kutman et al., 2010).

In plants, Zinc is predominantly present as low molecular weight complexes (e.g. in association with amino acids), storage metalloproteins, free ions and insoluble forms in cell walls. Complexation with organic ligands or phosphorus can inactivate zinc within the cells. The water-soluble zinc fraction in plants is considered to be the most physiologically active (Alloway, 2008).

Zinc has major physiological functions in plants:

- **Carbohydrate metabolism.**

  **A) Photosynthesis.** A lower number of chloroplasts was observed in zinc deficient plants (Bergmann, 1992)**.** This may be due to photooxidative damages depending on zinc nutritional status (Cakmak, 2000).

  **B) Starch formation.** Zinc deficiency influences many redox processes such as oxidation of cystein to cystine, which leads to a decrease in starch production (Bergmann, 1992)

- **Protein metabolism.** Zinc deficiency was shown to significantly depress protein synthesis and activity, because it an essential component of RNA and ribosomes (Marschner, 1986), it changes the distribution of the concentration of certain amino acids, it binds enzymes and substrates (Broadley et al., 2007; Bergman, 1992). Three

primary $Zn^{2+}$-ligand binding sites of protein are known: (i) structural Zn sites responsible for protein folding, where cysteine (Cys) is a major ligand, (ii) catalytic sites, and (iii) co-catalytic sites (Broadley et al., 2007).

- **Membrane integrity – oxidation protection.** Zinc deficiency induces the permeability of biomembranes (Bergmann, 1992) because it may be involved in the maintenance of ion transport, and in the maintenance of the membranes through the interaction with phospholipids and sulfhydryl groups (Alloway, 2008). It also increases the iron concentration, which stimulates the production of cell-damaging reactive $O_2$ species (Cakmak, 2000, Figure 7),
- **Auxin metabolism.** In Zn-deficient plants, the synthesis of the major growth hormone auxin may be inhibited (Alloway, 20008).
- **Reproduction.** Flowering and seed production is reduced in zinc deficient plants. Subnormal zinc concentration can provoke male sterility (Alloway, 2008).

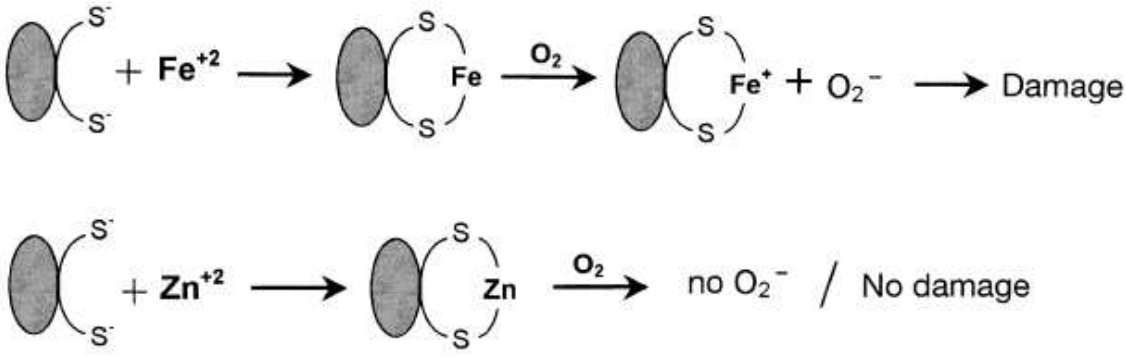



**Figure 7:** Zinc deficiency allows reactive $O_2$ species production due to higher iron availability. Fe binds to cysteine and is oxidised, while Zn doesn't react *(Cakmak, 2000)*.

Soil zinc is available to plants in the form of the free ions $Zn^{2+}$ and $ZnOH^+$, soluble organic complexes and labile zinc. Zinc uptake mainly takes place at the root-soil interface (Bergmann, 1992). At least 50% of the Zn that plant take up from the soil solution is $Zn^{2+}$, the remainder being bound to organic ligands (Broadley et al., 2007). *Poaceae* may transport zinc via complexes formed with non-protein amino acids, "phytosiderophores", which are released by the roots in the case of iron or zinc deficiency. The complexes are then transported to the cells with transport proteins (Alloway, 2008; Alloway, 2009). Root uptake, root-to-shoot

translocation and remobilisation (retranslocation) are the most important steps of Zinc transport (Erenoglu et al., 2011).

Zn mobility in plants is usually classified as low (Bergmann, 1992). However, Zn is more mobile than Mn, Fe, B or Mo but less than K or P in the phloem (Bergmann, 1992, Riesen and Feller, 2005). Its mobility in the phloem is termed "intermediate" (Moraghan et al., 1999). In maturing wheat, zinc moves from leaves to developing grains (Yang and Zhang, 2006), mature leaves acting as zinc sources. Developing wheat grains receive zinc from the flag leaf, i.e. the leaf situated the closest to the spike (Erenoglu et al., 2011).

Zinc has to cross at least two barriers to reach the wheat grain (Wang et al., 2011). Zinc and protein have been shown to be situated in the aleurone and embryo parts of the grain by co-localisation with protein and Zn staining (Kutman et al., 2010). Phytic acid, which is also situated in the aleurone layer of the wheat seed, is a strong chelating agent for Zn and other metal cations, making them unavailable for consumption (Regvar et al., 2011). These phytic acid complexes are called globoids. However protein is considered to be a zinc sink in the wheat grain, i.e. grain proteins could increase the storage capacities for Zn (Erenoglu et al., 2011, Kutman et al., 2010). This is supported by the positive correlations found between grain Zn and grain protein in wheat (Cakmak et al., 2004; Morgounov et al., 2007). Zinc transport, grain Zn accumulation and general protein concentrations in grains are increased with leaf senescence, possibly by remobilising Zn from senescing tissues (Moraghan et al., 1999, Kutman et al., 2010).

Zn is involved in many and different very important physiological functions in plants. This is why Zn deficiency has a major effect on wheat metabolism. Typical critical Zn concentrations for plant growth are between 18 and 20 mg $kg^{-1}$ in shoots, and 15 mg $kg^{-1}$ in grains (Alloway, 2009). In mature wheat grains, the critical Zn level is in the range of 5 to 10 mg $kg^{-1}$ (Reuter and Robinson, 1986). This induces a wide range of deficiency symptoms. In wheat, deficiency is visible when there is less than 10 mg Zn $kg^{-1}$, whereas visible toxicity symptoms appear at concentrations around 300 mg $kg^{-1}$ Zn (Broadley et al., 2007). However in the field, Zn deficiency is not visible uniformly. It appears during early growth stages (Follet and Westfall, 2004). Chlorosis, a common symptom for Zn deficiency, could also be caused by nitrogen or magnesium deficiency and is thus not conclusive proof of Zn deficiency. Other symptoms are stunted growth, prevented thickening, shortened internodes, few and small

fruits, a decrease in shoot growth and leaf size, light green colour of leaves (due to lower chlorophyll content), and white to brown necrotic patches on leaf blades (Koshgoftar et al., 2006, Bergmann et al., 1992). According to Bergmann (1992), the first typical symptoms are dot-like to blotchy spots that are first white and then brown and expand to cover the whole leaf. The network formed is irregular and younger leaves seem normal. Figure 8 shows an example of zinc deficient wheat leaves. On Figure 9, the colour and density difference of wheat grown with and without Zn fertiliser on a Zn deficient soil can be seen. In spring wheat, one phenotypic effect of adequate Zn fertilisation is to hasten maturity of heads on tillers, though without affecting the ultimate number of tillers (Moraghan et al., 1999).

**Figure 8:** Zinc deficiency of wheat leaves *(CIMMYT)*

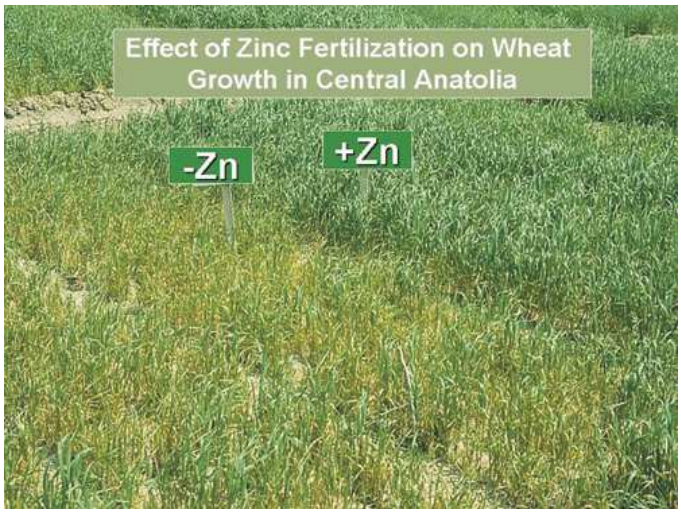



**Figure 9:** Effect on Zn fertiliser on wheat growth *(www.biokemi.org)*

Zinc deficiency in plant result from low Zn availability in soils. The extent of the deficiency also depends on the wheat genotype. Different wheat species and wheat varieties have also different "zinc efficiencies". Koshgoftarmanesh et al. (2009) define Zinc efficiency as "the ratio of yield (shoot dry matter or grain yield) produced under zinc deficiency to yield produced with zinc fertilisation". So-called "Zinc efficient genotypes" have a small response to Zinc fertilisation, whereas "zinc inefficient genotypes" largely react to Zn fertilisation. Zinc efficiency can vary according to differences in root uptake mechanisms: root architecture, presence of mycorrhizal fungi, release of phytosiderophores, proton exudation from roots etc. (Koshgoftarmanesh et al., 2009).

The effect of low soil zinc concentrations always depends on the presence of other macro- and micronutrients. For instance, trace elements are often located at protein binding sites and proteins are made of amino acids, which are mainly composed of N, O, and S. In many studies, nitrogen has been shown to have an influence on the effect of zinc in wheat. In durum wheat, Erenoglu et al. (2011) observed that N fertilisation was positively correlated with Zn uptake, Zn translocation, and Zn remobilisation from senescent and non-senescent leaves. This was explained by a possible increase of transporter proteins and nitrogenous chelators involved in these processes. In the same study, under Zn sufficient conditions, grain N and grain Zn concentrations were positively correlated. These observations were also made by Kutman et al. (2010), who suggested that, when Zn and N concentrations are sufficient, "N and Zn act synergistically in improving the grain Zn concentration" and highlighted that the selection of genotypes with high grain protein content could help improve wheat varieties

with respect to Zn accumulation. However Moraghan et al. (1999) did not observe any major effect of N fertilisation on grain-Zn concentration in spring wheat. They hypothesised that adding N fertiliser slowed down senescence of wheat leaves, which could decrease seed Zn-concentrations that are remobilised from senescing leaves.

One reason for these different experimental results might be the type of N-fertiliser used. Wang and Below (1998) observed that plant grown with $NO_3^-$ accumulated more Zn than those receiving $NH_4^+$ or a mix of nitrate and ammonium. Erenoglu et al. (2010) and Kutman et al. (2010) added $NO_3^-$, while Moraghan et al. (1999) used urea in their field experiment and a mix of $NO_3^-$ and $NH_4^+$in the greenhouse.

Zhu et al. (2001) found that, in soils with low phosphorus concentrations, a high P uptake after P fertilisation might reduce grain-Zn concentrations in *Triticum aestivum,* or even induce Zn deficiency. Reasons may be the dilution effect of increased shoot growth and a hindered Zn uptake by the roots and root-to-shoot transport due to a high P transport in the xylem. Zn bioavailability in plants is also dependent on the phytate concentrations, since phytate binds Zn (Alloway, 2009). It is thus important to know the P/Zn ratio, which should not be higher than 140 (Bergmann, 1992).

Adequate elemental sulphur application significantly increased the wheat grain uptake of Zn (Singh et al., 1986). A positive correlation was also found between grain Zn and grain S, as well as between grain Zn and grain Fe in spring wheat (Morgounov et al., 2007). In barley, Persson et al. (2009) observed the binding behaviour of Zn and Fe with P and S and stressed that both P and S were involved in Zn binding, but that iron was the main binder of phytic acid (containing P), while Zn was mainly bound to S-containing peptides in the grain tissue. It is thus a matter of the right combination of fertilisers. In that sense, Varavipour et al. (1999) obtained optimum overall yield and quality of wheat at a specific dose of P, S and Zn combined.

In the context of toxicity due to high salt concentrations, Koshgoftar et al. (2006) highlighted that Zn could partially counteract the negative effects of salinity on plant growth, with differences observed between genotypes. In all genotypes Zn fertilisation not only increased Zn concentrations, but decreased shoot concentrations of cadmium.

It appears that zinc could have optimal effects on wheat yields, quality and grain Zn content in the presence of carefully chosen amounts of other fertilisers, taking into consideration the genotype and the soil conditions.

### 2.4 Sulphur in soils

Sulphur is present in soils in organic and inorganic forms. Inorganic S compounds in soils are sulphate ($SO_4^{2-}$) and compounds of lower oxidation state such as sulphide ($S^{2-}$), thiosulphate ($S_2O_3^{2-}$), tetrathionate ($S_4O_6^{2-}$), polysulphides ($S_n^{2-}$, where n>10), sulphite ($SO_3^{2-}$), and elemental S ($S^0$). If the soil is well drained and well aerated, inorganic S occurs mainly as sulphate. Sulphate can be in the form of easily soluble fraction, adsorbed by soil colloids, insoluble, or co-precipitated with $CaCO_3$. Under anaerobic conditions, inorganic S mostly occurs as sulphide and elemental S. However, as for nitrogen, sulphur is mainly present in organic form, primarily in sulphur-ester linkages ("HI reducible S"), or directly bonded to carbon (Tabatabai, 2005a). Organic S generally represents 30 – 70% of soil S (Tandon, 2011). Figure 10 summarizes the classification of S – fractions in soils.

If soil S is present in organic form, mineralization processes of microbial nature will take place in order to transform it to inorganic sulphate with the help of enzymes such as arylsulfatases (Table 2). Elemental S in soil is oxidised to sulphate by microbial biochemical reactions. Depending on moisture, temperature, pH and nutrient availability, chemolithotrophs of the genus *Thiobacillus*, photoautotrophs (species of green and purple bacteria) and heterotrophical bacteria and fungi will oxidise elemental S (Tabatabai, 2005a). Once sulphate has been taken up by the plants, it is converted back to organic S, a process called assimilation (Table 2).

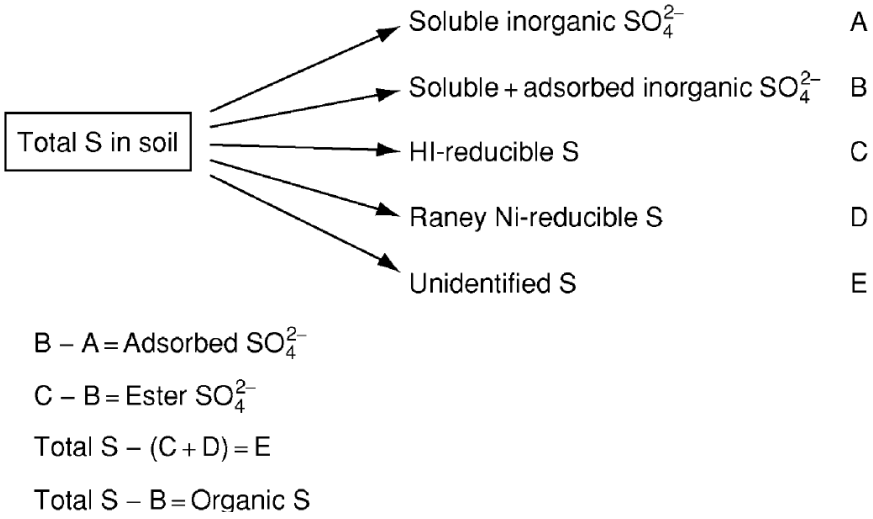



**Figure 10:** Organic and inorganic sulphur fractions in soil. S-containing organic compounds: C = Organic S that is not directly bonded to C and is reduced to $H_2S$ by hydriodic acid (HI); D = Organic S that is directly bonded to C (C-S) and is reduced to inorganic sulfide by Raney Ni; E = Organic S that is not reduced by either of the reagents *(Tabatabai, 2005a).*

Water-soluble as well as adsorbed sulphate is available to plants. Leaching of sulphate and sulphate adsorption in soils are influenced by chemical and physical parameters such as clay type and content, hydrous oxides, the presence of cations and other anions, soil pH, sulphate concentration, temperature, soil depth etc. Sulphate losses are reduced (i.e. adsorption is increased) by the presence of roots, of Al and Fe oxides or by increasing soil acidity (Tabatabai, 2005a). Adsorption of sulphate is negligible above soil pH 6.5 (Tabatabai, 1996).

Sources of soil sulphur are minerals, atmospheric depositions, and fertilisers (Tabatabai, 2005a). Two species of S are found in S-bearing minerals: sulphate, e.g. in gypsum ($CaSO_4$), or sulphide, e.g. as pyrite (FeS) or sphalerite (ZnS). Atmospheric S inputs can occur in the oxidised form as sulphur dioxide or sulphate, or in reduced forms such as $H_2S$. Natural atmospheric sources are volcanic activity, ocean spray, bacterial decomposition, and animal manure. Anthropogenic sources are combustion of S-containing fossil-fuels and gases released from industrial processes such as smelting and petroleum refining, which are deposited on the soil from the atmosphere.

**Table 2**: Relevant equations of the sulfur cycle in the plant-soil system (Tabatabai, 2005a).

| **Sulphur Cycle** | | |
|---|---|---|
| Mineralization of organic S | $2\text{Organic S} + 3O_2 + 2H_2O \rightarrow 2\ SO_4^{2-} + 4H^+$ | +2 |
| Assimilation of sulphate | $SO_4^{2-} + 8H^+ + 8e^- \rightarrow -SH_2 + 2H_2O + 2OH^-$ | -2 |
| Oxidation of $S^0$ | $2S^0 + 2H_2O + 3O_2 \rightarrow 2\ SO_4^{2-} + 4H^+$ | +2 |

$S^0$ oxidation in soils generates $H^+$ through sulphuric acid production (Table 2). Elemental S is thus used as an acidifying agent in order to change the solubility of certain elements (Bolan et al., 2005). For instance, the supply 100 mmol $S^0$ $kg^{-1}$ has been reported to decrease the pH from 7.2 to 6.8, which led to a 2.8 fold increase of the $Zn^{2+}$ solubility (Cui and Wang, 2005). The oxidation rate of $S^0$ is mainly influenced by temperature, pH, soil moisture and particle size of the fertiliser. A high specific area of $S^0$ fertiliser favours oxidation. After a lag phase probably explained by the multiplication and colonisation of microbial population, there is a period of maximum oxidation rate, followed by slower oxidation due to substrate depletion. Most soils have maximal oxidation rates at water potentials near field capacity (Chapman, 1989 and 1997). So low soil temperatures cause lower sulphur microbial mineralization rates (Anderson et al., 1992).

Generally, S deficiency is increasingly observed in agricultural soils all over the world. It usually affects regions with low S-fertiliser use and low atmospheric S inputs, i.e. regions far from towns, and is widespread where soils are light and precipitation high, inducing leaching of the mobile sulphate ion, such as in rainy tropical plantations. Sulphate adsorption is pH dependent, more $SO_4$ is bound in acid soils (Bergmann, 1992). In temperate and industrialised regions, S deficiency is becoming an issue in less remote areas because of the following reasons: i) increased use of fertilisers that contain less or no S as impurities, ii) more intensive cropping and increased yields depleting soil S reservoirs, iii) decreased use of S as a pesticide, iv) less combustion of coal and use of low-S-fuels (Tabatabai, 2005b).

Atmospheric sulphur oxides ($SO_x$) enter the soil through precipitation as sulphuric acid. Consequences of the so-called “acid-rain” are a corrosive effect on infrastructures, acid deposition on plants, surface water acidification, and soil acidification with its subsequent

metal toxicity issues (Bolan et al., 2005). This is why, in the last decades, air pollution is being more and more restricted by legislation and technical solutions (e.g. flue gas desulphurisation). This leads to a decrease of sulphur inputs by atmospheric deposition over industrialised countries, especially in Europe. Due to the reduction of S fertilisation as a positive external effect of pollution, intensively used agricultural land surrounding areas with a high industrial activity is now starting to be affected by S deficiency. Farmers need to increase bioavailable S concentrations of the soil. Fertiliser additions are one option to increase soil S concentrations. Since less and less sulphur is contained in fertilisers as impurities, it needs to be added separately. Common sulphur fertilisers are ammonium sulphate ($(NH_4)_2SO_4$), single superphosphate, ammonium phosphate sulphate, gypsum and elemental S, which is slowly converted to sulphate by microorganisms (Tabatabai, 2005b). $S^0$ also acts as a fungicide and has the effect of acidifying the soil, which increases the solubility of trace elements (Bolan et al., 2005).

Less information is available about S deficiency in developing countries. Fertiliser use and, thus, yields, are often very low, so biological S uptake from the soil may be low as long as yields are not improved. Some regions of the world are so sparsely populated that anthropogenic S inputs should be very low and S deficiency may appear if sulphate leaching is high. However around big towns, atmospheric pollution often resembles the situation a few decades ago in Europe and fertilisers probably have more S impurities than in developed countries. Information on pH, soil texture, precipitation, agricultural practices and distance to towns may allow one to estimate if S deficiency is expected. In over 300 districts in India, sulphur deficiency has been reported: 40-45% of 135000 soil samples were classified as S deficient, and another 30-35% as potentially S deficient (Tandon, 2011). This could be explained by the fact that agricultural land is used intensively in order to feed one billion people, by the low awareness of the S-deficiency issue, or by high sulphate leaching during the rainy season.

Typical total S contents of agricultural soils are in the range of 56 to 618 mg $kg^{-1}$ S in surface soils, and 40 to 200 mg $kg^{-1}$ S in subsoils (Anderson et al., 1992). In soils from humid and sub-humid regions, inorganic S represents only 2 to 6% of total S (Tabatabai, 2005b). The critical available soil sulphate concentrations for plants was set at 8 to 10 mg $kg^{-1}$ $SO_4$-S (or 24 to 30 mg $kg^{-1}$ $SO_4$) by Scott (1981), if the soil was extracted in $KH_2PO_4$. Since in field conditions, not only the soil, but also atmospheric deposition is a source of S for plants,

assessing plant available S in soils is difficult (Tabatabai, 2005b). Sulphate is also particularly dependent on moisture conditions and is easily leached, which can explain high variability in its soil content (Bloem et al., 2001). Advantages of using elemental sulphur as a fertiliser are that it is slowly released under the same conditions that are favourable to plant growth, and reduces leaching due to its insolubility. This is particularly true in coarse textured, i.e. well aerated soils (Chapman, 1997).

### 2.5 Sulphur in wheat

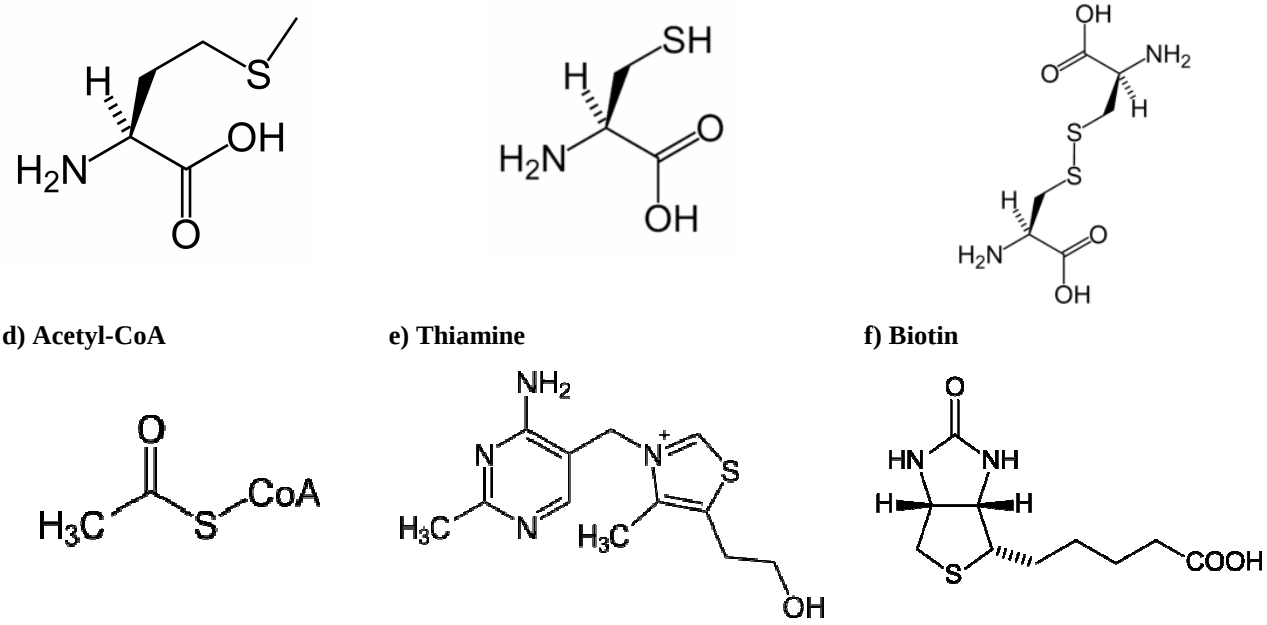
Sulphur is an essential element in plants. S is the fourth major plant nutrient after N, P and K and the third most widely deficient nutrient (Tandon, 2011). Plants take up sulphur mainly from soils, as sulphate ions that originate either from in-situ inorganic $SO_4$-S, adsorbed $SO_4$-S, or readily mineralisable soil organic S (Goh and Pamidi, 2003). Approximately 40% of the sulphate taken up by the plants is reduced to sulphide in order to be assimilated into organic S-compounds. The amino acids cysteine (CYS) and methionine (MET) (Figure 11) contain 90% of this incorporated S. However reduced S can be converted back to sulphate as a storage form. The S which is not needed can be stored in leaves, as well as in the seeds (Bergmann, 1992).

In all CYS- or MET containing compounds, S is either a structural component or it acts as a functional group (e.g. R-SH). It is thus involved in metabolic reactions because it is often located at the active sites of enzymes. Since it is required for the synthesis of the amino acids CYS and MET, S is a major constituent of plant proteins. S deficiency inhibits protein synthesis and induces high starch production. S is also contained in important coenzymes and prosthetic groups, such as ferredoxin, Acetyl-CoA, biotine (or vitamin H), thiamine (or vitamin B1), and is needed for the synthesis of chlorophyll (Marschner, 1986, Bergmann, 1992, Tabatabai, 2005b, Figure 11).

S is considered a rather immobile element in plants by Jones and Jacobsen (2001). But sulphate has been qualified as a rather mobile ion, which easily moves in the phloem, from root to meristem and young organs, and the transfer from older to younger leaves is easy (Bergmann, 1992). However, Eriksen and Mortensen (2002) stated that in S deficient conditions in barley, in contrast to N, S does not seem to be remobilised from vegetative tissue to younger leaves and grains in response to the S demand. The presence of available S

sources in the soil during grain filling is thus very important. The mobility of sulphur in wheat may depend on its form (sulphate or organic S), as well as the growth stage and S availability in soils.

**a) Methionine** **b) Cysteine** **c) Cystine**




**d) Acetyl-CoA** **e) Thiamine** **f) Biotin**

**Figure 11:** Important S-bearing molecules

Wheat plants contain 0.1 to 0.4% sulphur (Jones and Jacobsen, 2001). An adequate N/S ratio is very important for plant growth. In wheat, S is considered to be deficient if the N/S ratio is higher than 17 in young plants and 14.8 in grains. The critical S content was set to 0.12% in grains (Reuter and Robinson, 1986; Bergmann, 1992). In young shoots, the sufficiency level is higher, i.e. 0.21% (Tandon, 2011).

Typical visible S deficiency symptoms in wheat are a stunted and rigid erect appearance at early stage, smaller and narrower leaves, chlorosis, yellowing, poor tillering or delayed earing. It is difficult to distinguish from N deficiency symptoms, but generally S deficiency is first indicated in young leaves (Bergmann, 1992). Retarded growth, older leaves remaining green, reduced flowering and reduced grain filling, elongated internodes, leaves at an acute angle of the stem, and thin plants have also been reported in wheat (Tandon, 2011).

Many S deficiency symptoms in wheat resemble N deficiency symptoms, since N and S are the main constituents of proteins. According to Zörb et al. (2010), "N primarily affects protein concentration and biomass production, while S fertilization affects the fine tuning of storage protein composition." Because of an increasing N:S ratio, high N supply induces S deficiency. So in S deficient conditions, amino acids such as proline and glutamine are synthesized at the expense of cysteine and methionine. Less disulfide bridging provokes decreasing glutenin polymerisation in gluten proteins, reducing the nutritional and baking quality of flour (Figure 12). In S deficient conditions, priority is given to the synthesis of physiologically active proteins, while reducing S rich storage proteins mostly located in the grains (Zörb et al., 2010).

**Figure 12:** Bread baked with (left) and without (right) S-deficient wheat grains (left). *(http://praxisnah.de/index.cfm/article/5916.html)*

Various interactions between S and P have been reported. The effect depends on application rates. Plants have similar P and S requirements and P and S are adsorbed at similar sites on soil colloids and have similar precipitation schemes with Ca, Fe, and Al. At low to moderate rates of P application, the application effect on plants can be synergistic, while it may be antagonistic at higher P application rates (Tandon, 2011).

Since Zn is also involved in protein metabolism, it is very likely that Zn and S are tightly linked in many reactions. Both elements have catalytic functions due to their location at active sites of enzymes. For instance, Zn has a structural role of insuring protein folding by binding to cysteine (Broadley et al., 2007). In fact cysteine has a high association constant with Zn (9.2) and Zn exposure has been reported to increase sulphate transporters in maize and other plants (Na and Salt, 2010). In addition, –SH groups are powerful chelating agents for Zn transport (Kinnersley, 1993).

# 3 Materials and methods


**Figure 13:** Artificial catchment Hühnerwasser (Chicken creek) near Cottbus, Germany. ***(****Gerwin et al., 2010)*

## 3.1 Origin of the soil

The soil was collected on a post-mining landscape of the open cast mine Welzow-Süd near Cottbus, Germany (Figure 13). This sandy substrate originated from Pleistocene sediments deposited by glacier meltwater and was free of pyrite and free of coal particles. Thus

acidification due to weathering was not likely. In addition, no amelioration or planting measures had been carried out, so no soil development should have taken place (Gerwin et al., 2010). The dumped material was collected at a depth of 20 cm after 2 years.

## 3.2 Initial soil analysis

The substrate from Cottbus was oven-dried at 40°C and passed through a 2 mm sieve. Physico-chemical parameters were determined on the original dry material.

### *3.2.1 Soil texture analysis*

The grain size distribution was determined with the pipette method according to the reference methods of the Swiss federal agricultural research stations (FAL, RAC, FAW, 1996). 30g of the substrate was heated to 100 °C in a sand bath while 35% $H_2O_2$ was added in order to degrade the organic material. When no more reaction was visible (cessation of foaming), the material was dried on the sand bath and in the oven at 105 °C, and placed in a desiccator for cooling.

Five replicates of the dried substrate of 5 g each were put into beakers and a 0.2% Calgon solution was added. The particles were dispersed during 8 min by sonication (Labsonic U, Braun/Inotech, Dottikon, Switzerland). The material was put into a 500 mL graduated cylinder, containing 0.2 % Calgon solution, and shaken. Time measurement began as soon as no turbulence was visible in the cylinder. After 53 s and again after 2 h a 10 mL sample was removed from the cylinder with a pipette at a depth of 19 cm and put into a porcelain cup. The samples were dried at 105 °C and weighed. The mass of the blanks (dry cups with Calgon) was also determined. Soil texture was calculated from the weight difference between the blanks and samples (see formula in the appendix).

### *3.2.2 Organic carbon content*

The organic carbon content was determined by back-titration of dichromate according to the reference methods of the Swiss federal agricultural research stations (FAL, RAC, FAW, 1996).

The organic carbon content was calculated using the following simplified titration reaction:

$$4\ Cr^{6+} + 3\ C^{0} \rightarrow 4\ Cr^{3+} + 3\ C^{4+}$$

$$Cr^{6+} + 3\ Fe^{2+} \rightarrow Cr^{3+} + 3\ Fe^{3+}$$

As an organic carbon content of less than 2% was expected, 3 replicates of 2g of substrate were weighed into Erlenmeyer flasks. 10mL of potassium dichromate ($K_2Cr_2O_7$, 0.1667M), and 20mL of concentrated sulphuric acid were added to the substrate, reacted for 20 min and cooled. The solution was filtered after the addition of 150mL of pure water. Five drops of ferroin indicator were added to the replicates and two blanks. The excess dichromate solution was back titrated with ferrous ammonium sulphate solution ($Fe(II)(NH_4)_2(SO_4)_2 \cdot 6H_2O$, 0.2M) with the help of a titrator (Metrohm 775 Dosimat, Switzerland). The Fe(II) volume was noted down when the solution colour changed from blue-green to dark brown. Soil organic carbon and soil organic matter content were calculated using the consumed volumes of Fe(II) in the blank and in the sample (see formulas in the appendix).

### *3.2.3 Carbonate*

The $CaCO_3$ content was determined using a calcimeter (Eijkelkamp, Netherlands), which was first calibrated with pure calcium carbonate and then used to calculate carbonate content (FAL, RAC, FAW, 1996). The gas burette of the calcimeter was filled with water. In order to obtain a calibration curve, the volume of $CO_2$ produced by dissolution of $CaCO_3$ was determined twice for 0, 0.2g and 0.4g pure $CaCO_3$ by adding 7mL of 4M HCl to the reaction vessel and allowing the acid to react with the carbonate for 15min. To determine the proportion of calcium carbonate in the samples, the $CO_2$ volume produced with the same procedure for two replicates of 5g of homogeneous, ground, and dry substrate was inserted in the obtained calibration equation.

### *3.2.4 pH measurement*

The pH of the substrate was determined in 1:2.5 suspensions of pure water and 0.01 M calcium chloride (FAL, RAC, FAW, 1996). 25 mL of water or $CaCl_2$ were added to 10 g of substrate (three replicates for each solution). The samples were shaken three times, letting the particles settle in-between for 10 min. The pH was measured by dipping the electrode in the

solution, after having calibrated the pH-meter (Metrohm 691, Switzerland) with pH 7 and pH 4 solutions.

### *3.2.5 Total trace element content*

Total trace element contents were determined using X-Ray fluorescence (XRF) spectrometry (X-lab 2000 X-Ray Fluorescence, Spectro, Germany). Four grams of the ground substrate was weighed together with 0.9 g of wax into a beaker and homogenised by shaking with two polyamide balls for 8 min at $17s^{-1}$ with a MM200 mixer (Retsch, Germany). Then the samples were pressed into a tablet under a pressure of 15 T. Three replicates were analysed.

### *3.2.6 Total carbon and nitrogen*

Total carbon content and total nitrogen content of the substrate were measured by dry combustion using a CNS-2000 Analyser (LECO, US-Saint Joseph, Michigan).

### *3.2.7 Available sulphate*

Sulphate was determined by means of ion chromatography (Advanced Compact IC 86, Metrohm, Switzerland, Cloumn: Metrosep A supp 5 – 150/4.0, flow rate: 0.7 mL $min^{-1}$, eluent: 1.8 mM $Na_2CO_3$ and 1.7 mM $NaHCO_3$) after extraction with $KH_2PO_4$ (Zhao and McGrath, 1994). Five grams of dry substrate was weighed (3 replicates) and 25mL of 0.016M $KH_2PO_4$ added. The solution was shaken in an 'end-over end shaker' for one hour at 19 $min^{-1}$, centrifuged at 3500 rpm for 10 minutes, and filtered through ashless Whatman no. 589/3 filter paper. The calibration range was 1 - 50 mg $L^{-1}$ and the sample matrix was matched. Standards and samples were stored in the refrigerator and analysed within one day. For samples that were taken from unplanted pots over the duration of wheat growth, 5.5g of moist soil was extracted instead of 5g dry substrate as available sulphate is known to increase during drying (Tabatabai, 1996).

### *3.2.8 Available zinc*

Available Zn was measured using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES, Varian, VISTA-MPX, Germany) after extraction with diethylenetriaminepentaacetic acid and triethanolamine (DTPA-TEA). 20mL of a solution containing 0.005M DTPA, 0.01M $CaCl_2$, and 0.1M TEA, whose pH had previously been adjusted to 7.3, were added to 10g of air-dried soil (3 replicates). The samples were shaken on a horizontal shaker for two hours at 120 cycles $min^{-1}$, and filtered through ashless Whatman no. 589/3 filter papers (Reed and Martens, 1996). If they were not analysed within a day, samples were acidified with concentrated nitric acid and stored in the refrigerator until analysis. The calibration curve was established with seven points ranging from 0 to 1 ppm in the same matrix as the samples.

In addition to Zn, Cd, Cu, Fe, Mn, Ni and Pb were analysed in the extracts although the method was only developed to identify Zn, Cu, Fe and Mn (Reed and Martens, 1996). Fe and Mn concentrations should only be considered as rough estimations since they often exceeded the calibration range of 0 to 1 mg/L, while Cd measurements were below the detection limit. Table 3 shows the wavelength and detection limits of the analysed metals.

**Table 3:** Wavelength and detection limits of the analysed trace elements with ICP-OES

| **Element** | **Wavelength (nm)** | **Detection limit (ppb)** |
|---|---|---|
| Ni | 231.604 | 10 |
| Pb | 220.353 | 20 |
| Cd | 226.502 | 5 |
| Mn | 257.610 | 5 |
| Cu | 324.754 | 5 |
| Zn | 213.857 | 5 |
| Fe | 238.204 | 5 |

### 3.3 Pot Experiment

A factorial pot experiment was conducted in order to study the effect of sulphate or elemental sulphur on zinc concentrations in *Triticum aestivum* (spring wheat) grains of the variety Kavir. The factors where Zn and S; Zn levels were no added Zn and Zn (2 mg $kg^{-1}$) while the S levels were no added S, $SO_4^{2-}$ (100 mg S $kg^{-1}$) and $S^0$ (100 mg S $kg^{-1}$). Six treatments were tested: control, zinc (Zn), elemental sulphur ($S^0$), sulphate ($SO_4$), zinc + elemental sulphur ($Zn+S^0$), and zinc + sulphate ($Zn+SO_4$).

1 kg of dried soil per pot was thoroughly mixed with fertiliser solutions containing N, P and K, Zn, and sulphate as described in Table 4. Elemental sulphur was added as a powder. After mixing the soil, deionised water was added to bring the soil up to 80% field capacity (105g water per kg dry soil). The soil was then transferred to 1L plastic pots.

All pots received the same quantity of added nitrogen, phosphate and potassium. Nitrogen was added as nitrate rather than ammonium, because Wang and Below (1998) showed that $NH_4^+$ fertilisers could have inhibitory effects on Zn accumulation. Moreover, ammonium may induce a fall in pH of the substrate (Marschner, 1986). Potassium was added either as KCl or as $K_2SO_4$, depending on the treatment. Due to a calculation error at the beginning of the experiment, the treatments with sulphate only received 78% of the intended 100mg of $SO_4$-S. The remaining 22% were added one week after planting as a solution. The sulphur and sulphate additions were equivalent to 100 mg S $kg^{-1}$. The Zn addition was 2 mg Zn $kg^{-1}$.

The *Triticum aestivum* seeds were allowed to germinate on filter paper soaked with deionised water inside opened Petri dishes. After one week, two seedlings were planted per pot. Three weeks after planting, the less developed wheat plant of the two was removed from each pot, leaving a single wheat plant. Each treatment consisted of 5 planted pots and also had 1 unplanted pot where the evolution of pH, available sulphate and Zn, independent of plant growth factors such as root exudation, was monitored by monthly sampling.

The soil moisture content in the pots was maintained at approximately 80% field capacity by weighing and adding deionised water. The plants were watered every day during the first two weeks, and every second day until physiological maturity was reached by the majority of the

wheat plants. The position of the different pots followed a totally randomised configuration and the wheat was grown in a climate chamber with conditions as in Table 5.

Pictures of the wheat plants were taken every week and parameters allowing determining the most important growth stages and the "health" of the wheat were documented. The observations focussed on the number, colour, and size of leaves, tillers, nodes, boots, heads, and flowers. Leave damages and other deficiency symptoms were also documented.

**Table 4:** Fertiliser treatments of the pot experiment

| **Treatment** | **Added fertiliser amounts *** | | | | |
|---|---|---|---|---|---|
| ***Control*** | 200 mg N $kg^{-1}$ | 100 mg P $kg^{-1}$ | 244 mg K $kg^{-1}$ | - | - |
| ***Zn*** | 200 mg N $kg^{-1}$ | 100 mg P $kg^{-1}$ | 244 mg K $kg^{-1}$ | 2 mg Zn $kg^{-1}$ | - |
| ***$S^0$*** | 200 mg N $kg^{-1}$ | 100 mg P $kg^{-1}$ | 244 mg K $kg^{-1}$ | - | 100 mg $S^0$ $kg^{-1}$ |
| ***$SO_4$*** | 200 mg N $kg^{-1}$ | 100 mg P $kg^{-1}$ | 100 mg $SO_4$-S $kg^{-1}$ and 244 mg K $kg^{-1}$ | - | - |
| ***Zn+ $S^0$*** | 200 mg N $kg^{-1}$ | 100 mg P $kg^{-1}$ | 244 mg K $kg^{-1}$ | 2 mg Zn $kg^{-1}$ | 100 mg $S^0$ $kg^{-1}$ |
| ***Zn+ $SO_4$*** | 200 mg N $kg^{-1}$ | 100 mg P $kg^{-1}$ | 100 mg $SO_4$-S $kg^{-1}$ and 244 mg K $kg^{-1}$ | 2 mg Zn $kg^{-1}$ | - |

*N was added as $Ca(NO_3)_2.4H_20$; P was added as $Ca(H_2PO_4)_2.H_20$; $SO_4$-S was added as $K_2SO_4$, $S^0$ was added as powder. If no $K_2SO_4$, K was added as KCl; Zn was added $ZnCl_2$.

**Table 5:** Climate chamber conditions

| **Time (h)** | **Temperature (°C)** | **Relative humidity (%)** | **Light (%)** | **Light intensity (lux)*** |
|---|---|---|---|---|
| <0.1 | 22 | 70 | 0 | at pot height: 11505±161 |
| 0.5 | 22 | 65 | 80 | 30 cm height: 16439±263 |
| 14 | 22 | 65 | 80 | |
| 0.5 | 22 | 70 | 0 | |
| 9 | 15 | 70 | 0 | |

* Mean ± SE (N = 36 planted, N = 30 unplanted soils)

Approximately once per month, the unplanted pot of each treatment was sampled. PH, available sulphate and DTPA extractable Pb, Cd, Mn, Cu, Zn, and Fe were analysed in order to follow the evolution of these parameters with time.

### 3.4 Soil sampling and analysis

Soil sampling and analysis of unplanted pots during wheat growth and of all pots after harvest were conducted in the same way. After wheat harvest, the soil was left in the pots at 80% field capacity for one day before sampling soil cores with 50 mL plastic PE-tubes. Thus the soil was kept in the pots during 120 days.

For available sulphate analysis, 5.5 g moist soil was extracted immediately after sampling as described previously. After sampling, the extract was additionally filtered through 0.45µm syringes. Although Tabatabai (1996) recommended to measure available sulphate within 24h after sampling, due to technical problems these soil extractions were kept in the refrigerator and, since no precipitation was observed, they were analysed after 2 days.

The remaining sampled soil was oven dried at 40°C. Soil pH measurements in $CaCl_2$, DTPA-TEA extractions and available Cu, Fe, Mn, Ni, Pb and Zn analysis in the ICP-OES were conducted as described above.

### 3.5 Plant sampling and analysis

Wheat plants were harvested 16 weeks after planting. Shoots were cut at less than 1 cm of the soil surface. Roots were not collected. Plants were carefully rinsed first with deionised and then with ultrapure water to remove potential contamination. Wheat heads were separated from the rest of the shoots, so that in this study “shoots” refers to the above-ground part of a plant without the heads. Wheat heads and shoots were stored in paper bags and dried at 60°C in a drying oven during one week. Weights were determined before and after drying respectively. After drying, the grains were manually removed from the husks. Grains were ground using a MM200 mixer mill (Retsch, Germany), while all other above-ground wheat parts were ground with a high-speed rotor ZM 200 mill (Retsch, Germany).

About 200 mg (exact weight noted) of dry ground wheat shoots and the chaff of the wheat heads of the $SO_4$ treatment were weighed into Teflon tubes for wet digestion. After addition of 15 ml $HNO_3$ (65%), the plant tissue was covered with disposable (PE) watch glasses and digested for 90 min in a DigiPrep MS Block Digestion System (SCP Science). The samples were cooled down for 30 min. Then 3 ml of $H_2O_2$ (30%) was added and the procedure was repeated. Finally, the digested samples were transferred into 50mL PE-tubes, rinsed three times with ultrapure water and then filled with ultrapure water till the 50mL mark.

Approximately 200mg of dry milled grains were weighed to teflon bombs. A mixture of 2 ml $HNO_3$ (65%) and 4 ml of $H_2O_2$ (30%) was added and the bombs were left under the fume hood for 30 min covered with loose lids. The samples were then digested in a Microwave Digestor (IavisETHOS, MLS, Germany). After cooling, the digest was also transferred into 50mL PE-tubes, the bombs rinsed three times with ultrapure water, and then the tube filled with ultrapure water till the 50mL mark.

The elements Zn, S, P, Mn, Mg, K, Fe, Cu, and Ca were analysed with ICP-OES. Two calibrations curves were established. For Ca 396.8, Cd 226.5, Cu 324.8, Fe 238.2, Mg 279.6, Mn 257.6 and Zn 213.9, the curve was established out of six points ranging from 0 to 100 µg $L^{-1}$. For K 766.5, P 213.6, and S 182.0, the calibration curve was made with 6 point ranging from 0 to 10 mg $L^{-1}$. Standards contained the stock solution, 10mL $HNO_3$ (65%), and were filled with nanopure water the 50ml mark of the PE tube.

For quality assessment we analysed IPE plant reference material of *Triticum aestivum* grains (N=2) and *Oryza sativa* straw (N=1), as well as 5 replicates of our plant material in the ICP-OES.

### 3.6 Statistical analysis

Treatment effects were determined using analysis of variance (2-way ANOVA) in combination with post hoc analysis by Tukey tests using SPSS (version 19, IBM SPSS Statistics, Chicago, USA).Table 6 shows which variables were transformed in order to have normally distributed residuals. Differences with $p < 0.05$ were considered significant.

**Table 6:** Transformation of variables for 2-way ANOVA

| **Variable** | **Transformation** | **Variable** | **Transformation** |
|---|---|---|---|
| Soil [$SO_4$] | square root | Total Zn in grains | none |
| Soil [Zn] | none | Total S in grains | none |
| Soil [Mn] | Log10 | Grain [Zn] | none |
| Soil pH | none | Grain [S] | Log10 |
| Total dry biomass | none | Grain [P] | none |
| Shoot biomass | Log10 | Grain [Mn] | none |
| Grain biomass | none | Shoot [Zn] | Log10 |
| Seed number | none | Shoot [S] | Log10 |
| Total Zn in shoots | none | Shoot [P] | Log10 |
| Total S in shoots | Log10 | | |

# 4 Results

## 4.1 Initial soil characterisation

In this experiment, wheat was grown on a substrate originating from a sandy substrate that was used to make an artificial catchment. Table 7 gives the properties of the untreated dry soil. The results of our soil characterisation are in good accordance with Gerwin et al. (2010) soil characterisation of a location in the same research area. With a very high sand fraction (89%), and very low clay content (2%), the substrate texture was classified as sand according to US Soil Taxonomy. This implies a low cation exchange capacity (Jones and Jacobsen, 2001). The pH was slightly alkaline (7.56 in $CaCl_2$), inducing a low bioavailability of cations such as $Zn^{2+}$ (Marschner, 1986), whereas anions such as sulphate are mobile due to their negative charge. Since almost no biological activity had taken place in the substrate before the experiment, the substrate was very low in organic matter (0.3%), and poor in total nitrogen with less than 0.01%, which is one order of magnitude lower than total nitrogen concentrations usually encountered in agricultural soils (Scheffer and Schachtschabel, 2002). The material had a concentration of 0.19 mg/kg DTPA-extractable zinc. It was thus Zn deficient (critical available Zn concentration for wheat: 0.5. to 1.5 mg/kg, Alloway, 2009). The available sulphate concentration of 7 mg $SO_4$ $kg^{-1}$ was also very low compared to the critical range for crop supply of 24 to 30 mg $SO_4$ $kg^{-1}$ (Scott, 1981). The calcium carbonate content of 1% is classified as low (FAL, RAC, FAW, 1996). Table 7 also shows total concentrations of sulphur and a selection of heavy metals from the XRF analysis.

**Table 7:** Physico-chemical soil parameters of the substrate/soil used in the experiment. Mean values and standard error (N = 3).

| **Parameter** | | **Mean** | **Standard error** |
|---|---|---|---|
| pH (water) | | 8.86 | 0.01 |
| pH ($CaCl_2$) | | 7.56 | 0.01 |
| Texture | % clay | 2.3 | 0.2 |
| | % loam | 8.8 | 0.2 |
| | % sand | 88.9 | 0.3 |
| Organic matter (%) | | 0.29 | 0.01 |
| Carbonate (%) | | 1.08 | |
| DTPA extractable zinc (mg/kg) | | 0.19 | 0.03 |
| Available sulphate (mg/kg) | | 7.21 | 1.03 |
| Total nitrogen (%) | | 0.00925 | 0.001038 |
| Total sulphur (mg/kg) | | 95.5 | 14.0 |
| Total Zinc (mg/kg) | | 16.0 | 0.98 |
| DTPA extractable copper (mg/kg) | | 0.28 | 0.0026 |
| DTPA extractable iron (mg/kg) | | 3.87 | 0.1734 |
| DTPA extr. manganese (mg/kg) | | 1.88 | 0.0360 |
| DTPA extractable nickel (mg/kg) | | 0.05 | 0.0044 |
| DTPA extractable lead (mg/kg) | | 0.17 | 0.0057 |

## 4.2 Soil analysis

In soils with plants, contrary to expectations, the pH slightly increased after sulphur additions (Figure 14). Compared to the control treatment, the increase did not exceed 0.23 pH units. In both $S^0$ treatments, the pH increased less than in $SO_4$ treatments, but this was not significant. All soils without plants had a lower pH than soils with plants. On non-planted soil, the $S^0$ and Zn+ $S^0$ treatments show lower pH values than the control treatment.

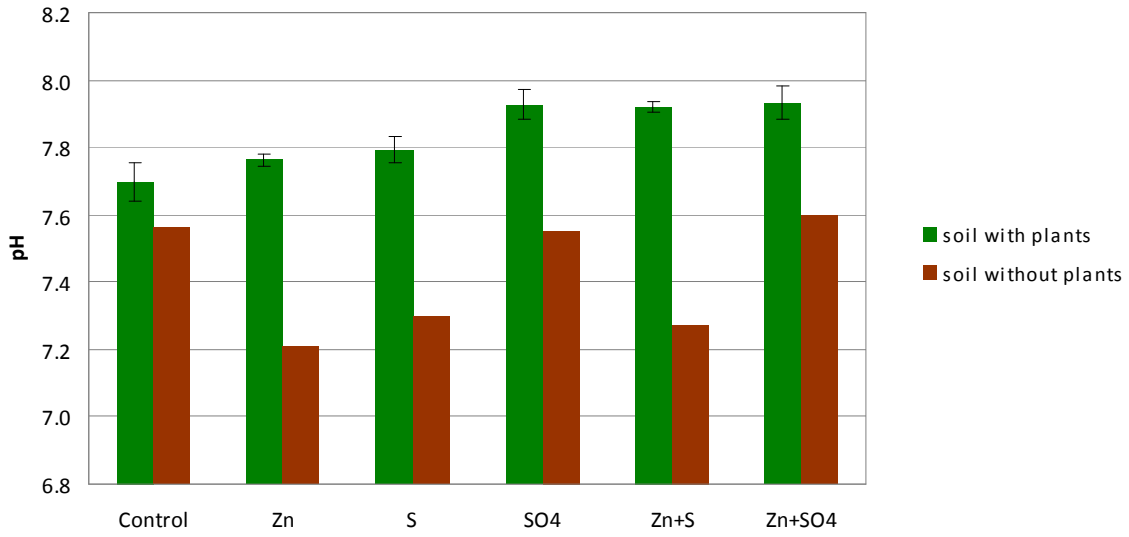



**Figure 14**: Soil pH ($CaCl_2$) after wheat harvest. Mean ± SE (N = 5 planted, N = 1 unplanted soils).

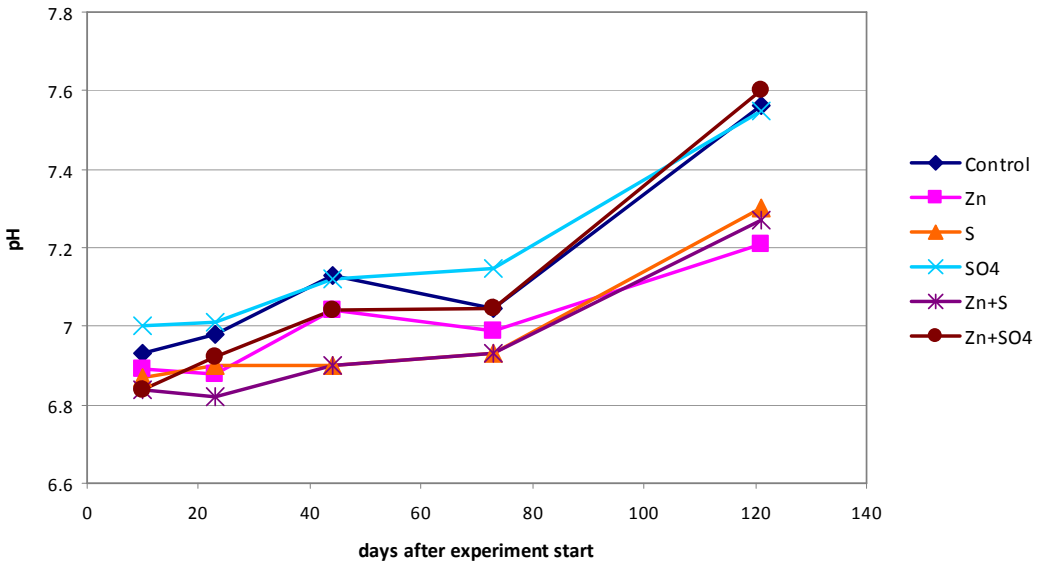



**Figure 15:** pH ($CaCl_2$) of unplanted soils with time. On the first day, only 77 mg $SO_4$ was added to the sulphate treatments. An additional 23 mg were added 16 days later.

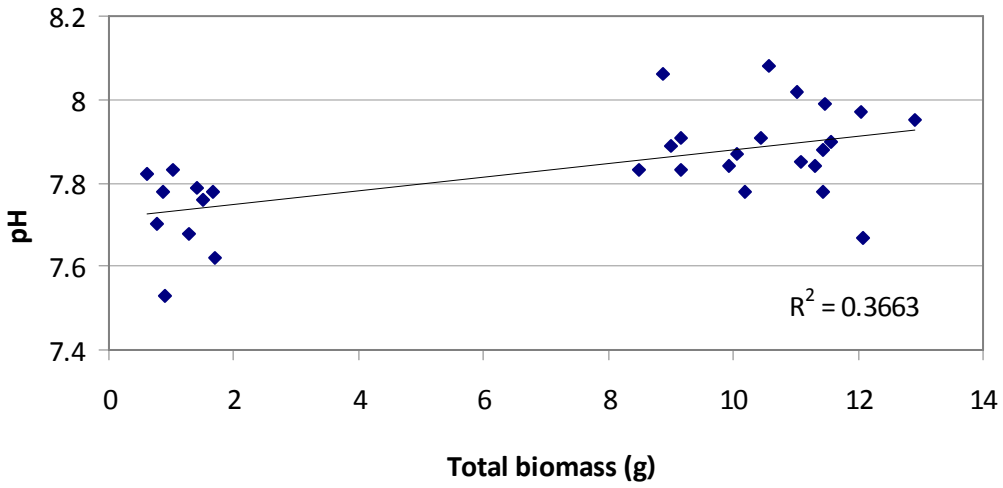



**Figure 16:** Soil pH at harvest vs. total above ground dry biomass of wheat

The pH of pots without plants was measured over the time of the experiment (Figure 15). For all treatments, pH increased over time. Addition of fertiliser initially reduced pH ($CaCl_2$) for all treatments. The control, $SO_4$ and $Zn+SO_4$ treatments at harvest time had a pH similar to unfertilised initial soil, i.e. 7.56. $S^0$ and $Zn+S^0$ treatments always had a lower pH than $SO_4$ and $Zn+SO_4$ treatments. But since the Zn treatment follows the same pattern, it may not have been related to sulphur oxidation. This is in contradiction to the expected effect of a pH decrease due to additions of $S^0$. In Figure 16, pH vs. total plant biomass can be seen. Two clusters can be observed: soils where plants developed only low biomass have a slightly lower pH than plants with higher biomass. The cluster with low biomass corresponds to control and Zn treatments, while the cluster with high biomass corresponds to treatments with added sulphur.

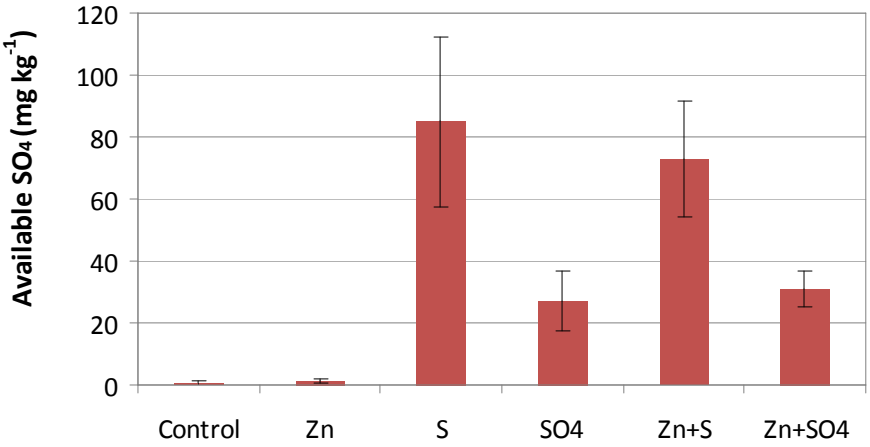



**Figure 17:** $KH_2PO_4$ extractable available sulphate in planted soils after harvest.
Mean ± SE, N = 5. Initial addition: 299 mg $SO_4$ per pot.

Figure 17 shows the soil concentrations of available $SO_4$ in planted soils after harvest. Concentrations in soils significantly increased if any form of sulphur was added ($p<0.001$). Elemental sulphur additions resulted in significantly higher available sulphate concentrations after harvest than sulphate additions ($p<0.05$). Zinc additions did not affect available sulphate concentrations in soil. The total sulphur content of the substrate wheat was grown in was 95.5 mg. $kg^{-1}$. The critical soil sulphate level for plant growth is 8 to 10 mg $SO_4$-S per kg dry soil, i.e. 24 to 30 mg $SO_4$ $kg^{-1}$. According to the very low sulphate concentrations in the control and Zn treatments, the original substrate was nearly free of available sulphate. Most of the available $SO_4$ in treatments with added sulphur should thus originate from the 100 mg $kg^{-1}$ dry soil added $S^0$ or $SO_4$-S.

Available sulphate was measured over time in the unplanted pots. The results were very heterogeneous, although the $SO_4$ solution and $S^0$ were well mixed and the $S^0$ powder was very fine. In the $S^0$ and Zn+$S^0$ treatments, it can be seen that the available sulphate in soil slowly increased with time (Figure 18). The last point of the Zn+$S^0$ treatment may be explained by the fact that only one replicate was used and a $S^0$ grain may have been hit.

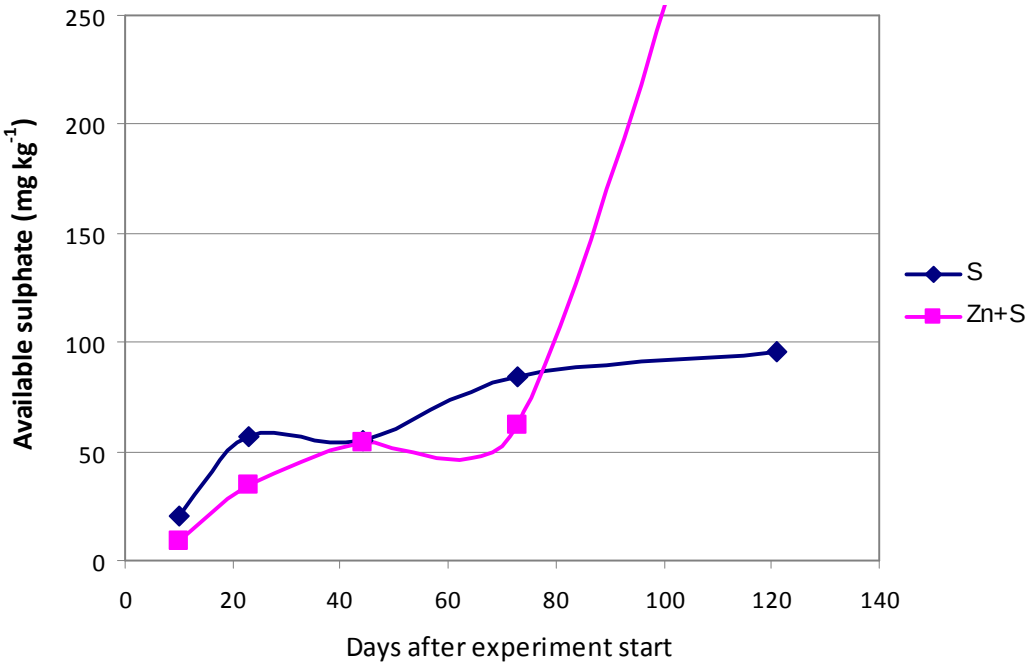



**Figure 18:** Available $SO_4$ in the $S^0$ and Zn+$S^0$ treatments with time. Initial addition: 299 mg $SO_4$ per pot.

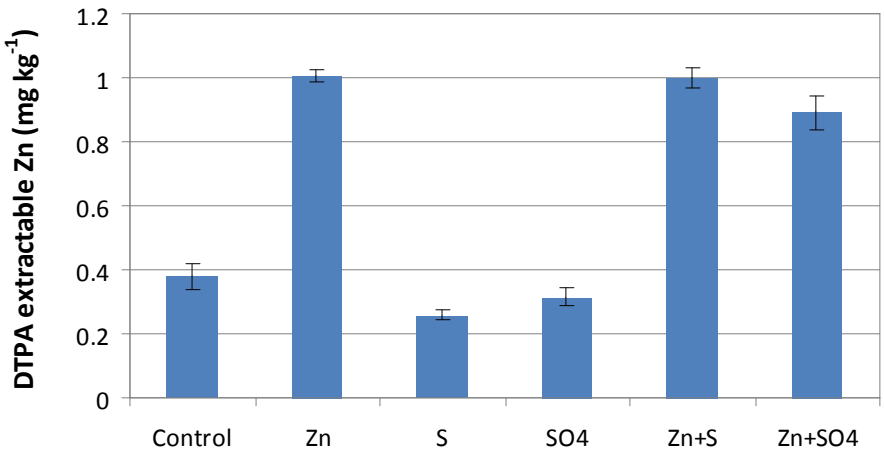



**Figure 19:** DTPA extractable zinc in planted soils at harvest. Mean ± SE, N = 5. Initial addition: 2 mg Zn per pot.

Zinc ($p<0.001$) and sulphur ($p<0.05$) had a significant effect on the DTPA-extractable Zn concentrations in planted soils after harvest (Figure 19), but $S^0$ and $SO_4$ were not significantly different. Zn amendments increased the available zinc in soils, while sulphur inputs decreased them to a lesser extent. This contradicts the initial hypothesis that the addition of elemental

sulphur would lower the pH and therefore increase the available Zn. Available Zn was also measured over time in the unplanted pots, but no change was observed.

Other DTPA-extractable metal contents were measured simultaneously to zinc. Sulphur ($p<0.001$) had highly significant effects on manganese concentrations in soil (Figure 20): the addition of elemental sulphur significantly increased soil manganese concentrations ($p<0.001$) in contrast to sulphate or zinc additions, which had no specific effect.

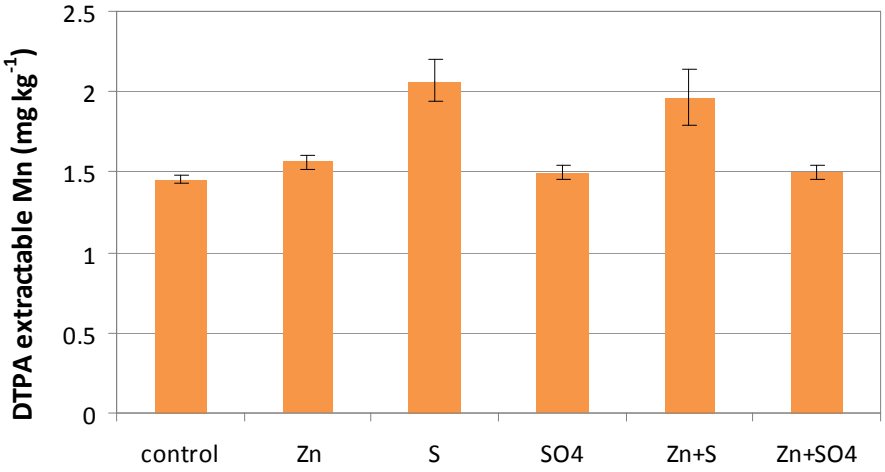



**Figure 20:** DTPA-extractable manganese concentrations per treatment in planted soils after harvest. Mean ± SE, N = 5

### 4.3 Wheat growth and biomass

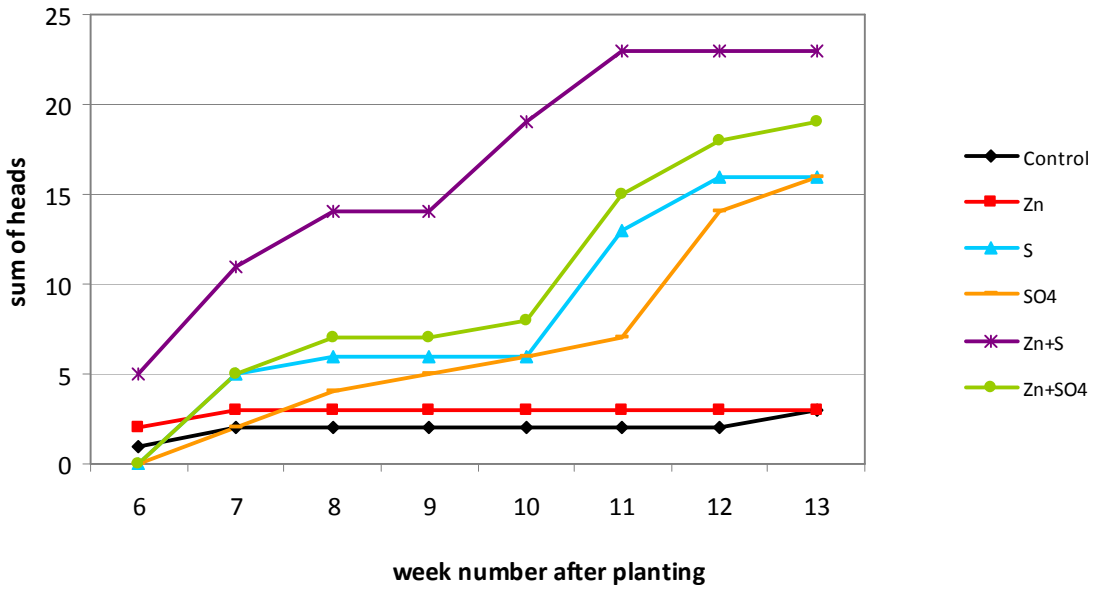



**Figure 21:** Total number of wheat heads per treatment (sum of 5 pots) with time. Heading began during week 6.

In the wheat plants, many differences between treatments were observed at harvest and also during wheat growth. Usually in plant experiments the time needed to attain important growth stages such as tillering, booting, heading, anthesis and physiological maturity of grains is compared. However in this experiment, plant development took place in two phases which will be analysed: a first generation of wheat heads and seeds developed before the 11$^{th}$ week, while a second generation of wheat heads only started to develop at this time. In most of the treatments, this phenomenon happened at the same moment in pots that had not yet gone through the transition between vegetative and generative growth, as well as in pots that had already started generative growth, but developed "new" spikes that were generally smaller. Figure 21 gives an overview of the two growth phases. The Zn+$S^0$ treatment started its second phase earlier and already had more spikes present. In the control and Zn treatments, some tillers developed but did not reach the heading stage. $S^0$, $SO_4$ and Zn+$SO_4$ treatments followed a very similar trend.

As can be seen in Figure 22, at the end of week 7 approximately half of the plants had developed wheat heads and the older leaves were beginning to dry, while in the other pots plants had developed many tillers without any visible sign of ear development. In the control

and Zn treatments, there either were plants that had tillered but had no heads, or plants with a single stem and an ear. Both treatments looked very similar and had the common pattern of very light coloured leaves. Elemental sulphur, sulphate, and sulphate plus zinc treatments had developed wheat ears in only two pots, while from five pots treated with $S^0$+Zn, four plants already had spikes. The spikes of the two $SO_4$ treatments were closely surrounded by the still erect flag leaf, whereas in both $S^0$ treatments, the ears were entirely visible and the flag leaves were pointing down. During week 11 however (Figure 23), wheat tillers that had seemed infertile before started to differentiate: some tillers dried up while other developed boots and, later, heads. In control and Zn treatments, plants tried to differentiate into the generative state, i.e. the shoot apex developed a darker colour and a flag leaf seemed to develop. All S treated plants having received either form of S developed boots and spikes. Where $S^0$ was added, boots opened faster.

At harvest time (week 16), as shown in Figure 24, all treatments that had received either $S^0$ or $SO_4$ had visibly developed more biomass and more spikes than the control and Zn treatments. Differences between treatments were still apparent, though. Only in the Zn+$S^0$ treatment, all parts of the plant were completely dry. In all other treatments, some parts of the plants were still green and new tillers, even new boots were developing. Also some grains were still green when they were harvested. In the $S^0$, $SO_4$ and Zn+$SO_4$ treatments respectively, a single "mutant" head appeared, in which spikes came out instead of seeds. In addition, in $SO_4$ treated plants, more microbial activity was observed in the soil (presence of green and red algae), and in the $SO_4$ treatment, every plant had irregular dark spots on all plant parts, which may have indicated the presence of a fungus.

Zn+$S^0$ treated plants had a smaller mean plant height at harvest time than the other plants having received any type of S addition, but were taller than the control and Zn treatments. Table 8 gives the average number of seeds per pot and seeds per head, comparing the seed number per head from the first and second generation. It can be seen that spikes of plants that developed before the $11^{th}$ growth week produced 3 times more grains or 3.6 times more seeds per single wheat head than "late spikes". Many heads of the second generation had almost or totally empty hulls. Indeed some heads did not produce any flowers (thus no grains). Table 9 represents the total number of heads, the total numbers of heads that reached anthesis and their proportion per treatment. Plants that received both $S^0$ and Zn not only had developed

more spikes than the other treatments, but all of them reached anthesis. The earlier a spike was developed, the more flowers could be observed (pictures and data not shown here).

**Table 8:** Comparison between early and late developed heads (number of seeds per plant and per head).

| | **Early wheat heads** | | **Late wheat heads** | | **Early/Late** |
|---|---|---|---|---|---|
| | Mean | Standard error | Mean | Standard error | Mean |
| **Number of seeds/plant** | 81 | 13.4 | 26 | 7.8 | 3.0 |
| **Number of seeds/head** | 27 | 2.9 | 7 | 2.4 | 3.6 |

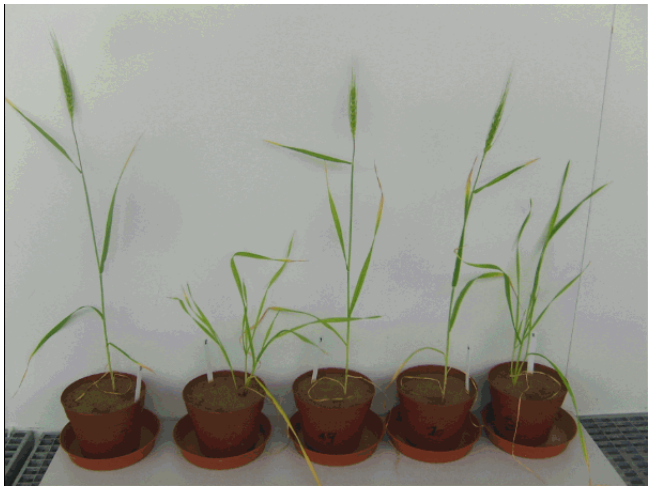
**Table 9:** Total number of heads and number of heads that reached anthesis in each treatment

| | **Total number of heads** | **Heads that reached anthesis** | **Fraction (%)** |
|---|---|---|---|
| **Control** | 3 | 2 | 67 |
| **Zinc** | 3 | 3 | 100 |
| **Elemental sulphur** | 16 | 16 | 100 |
| **Sulphate** | 16 | 13.5* | 84 |
| **Zn+$S^0$** | 23 | 23 | 100 |
| **Zn+$SO_4$** | 19 | 17 | 89 |

**Flowers were only partially developed*

26.4.2011 (end of week 7)

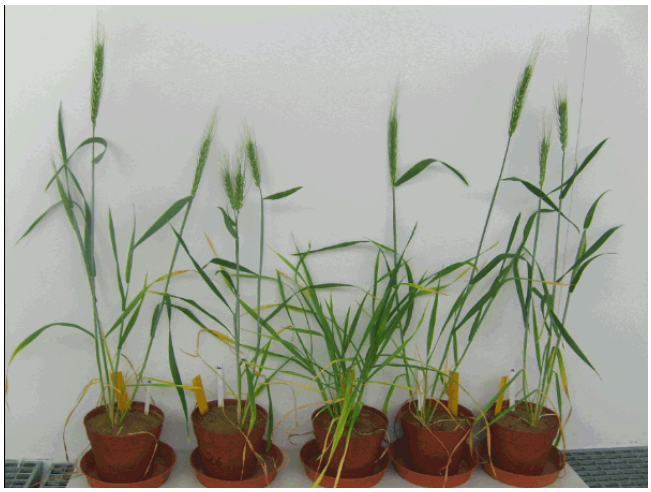
Control



Zinc

Sulphur

Sulphate

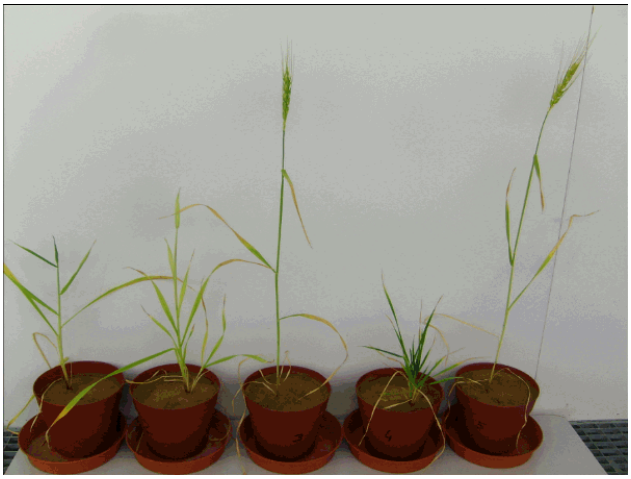


Sulphur and zinc

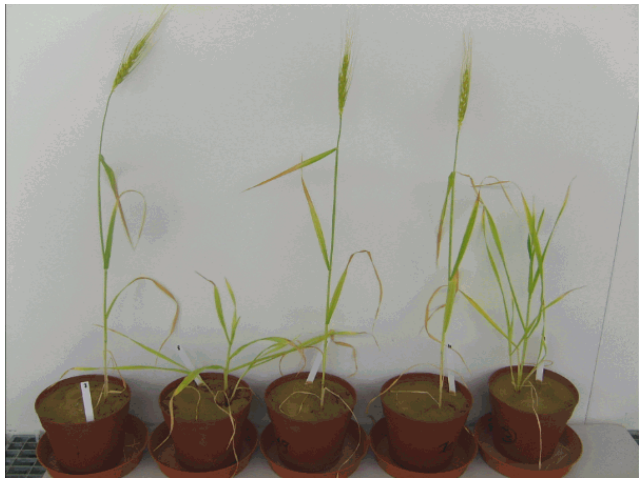
Sulphate and zinc

**Figure 22:** Wheat plants at the end of the week 7.

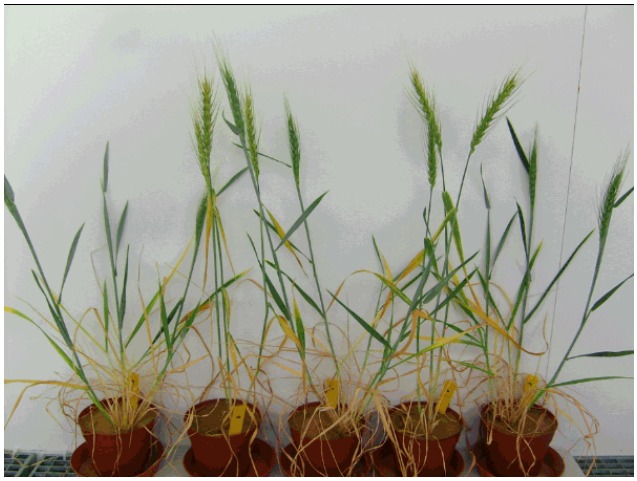
23.5.2011 (week 11)



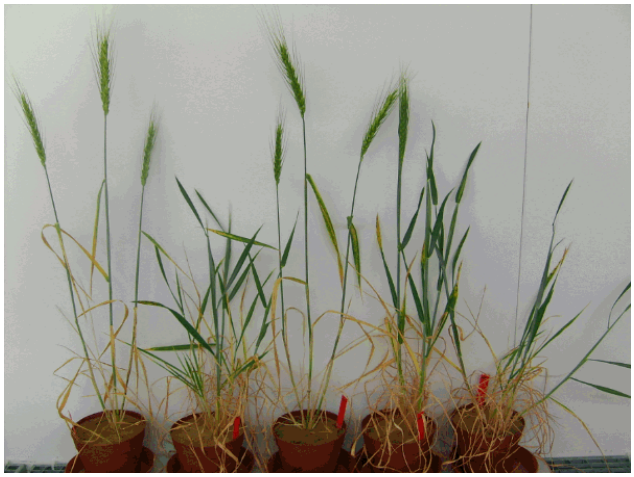
Control



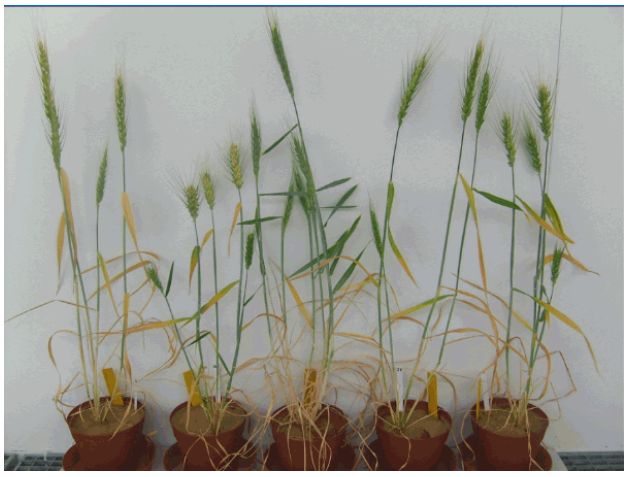
Zinc



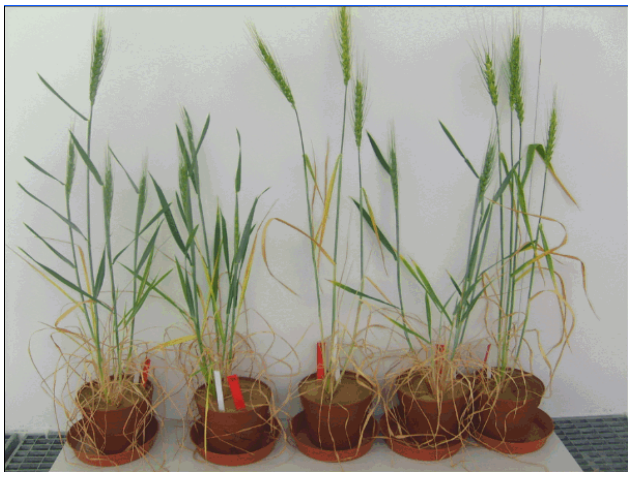
Sulphur



Sulphate



Sulphur and Zinc

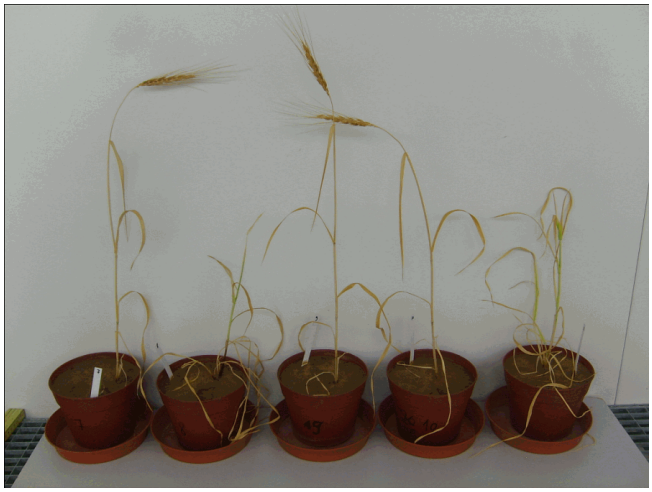


Sulphate and Zinc

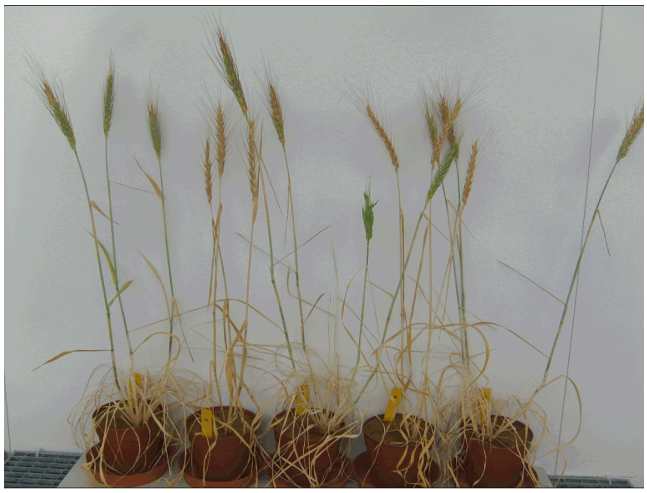
**Figure 23:** Wheat plants at the end of week 11

28.06.2011 (week16)

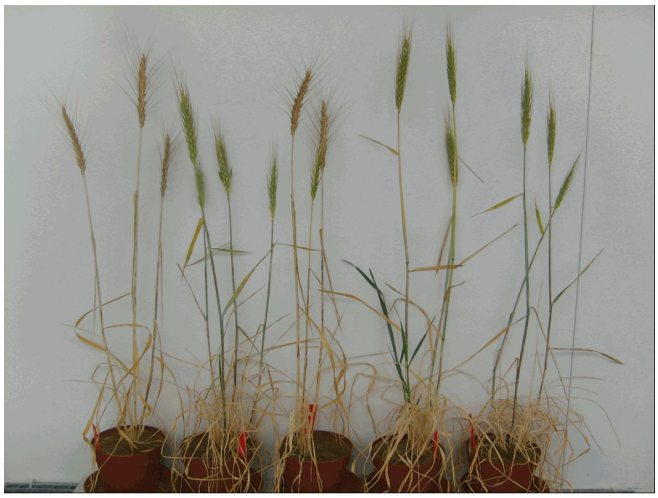
Control



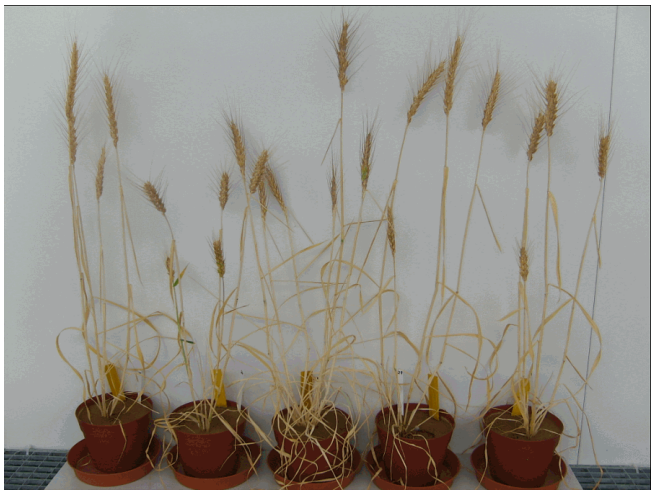
Zinc



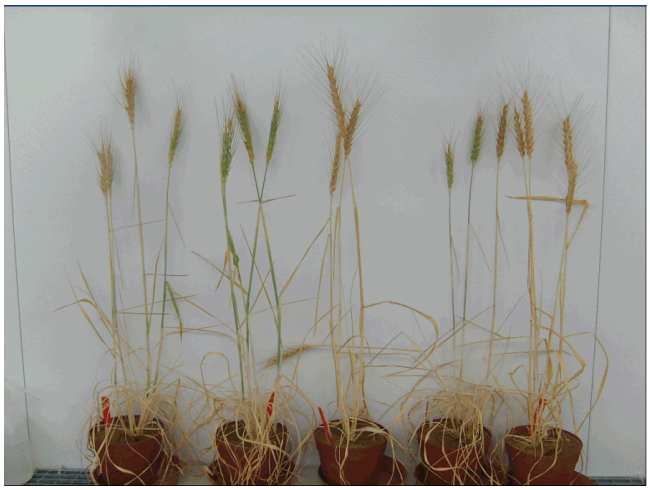
Sulphur



Sulphate



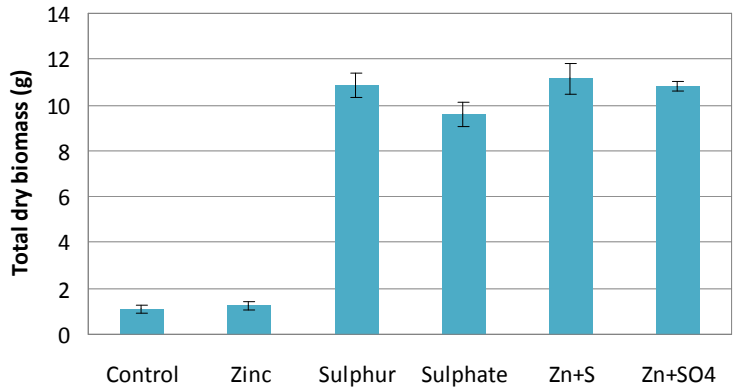
Sulphur and zinc



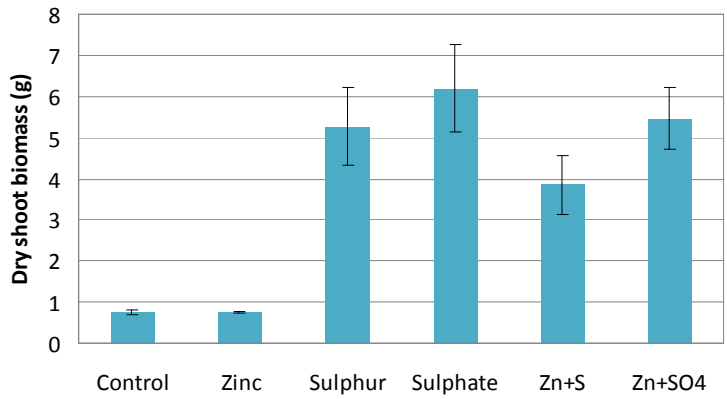
Sulphate and zinc

**Figure 24:** Wheat plants on harvest day (week 16).

a)




b)




c)

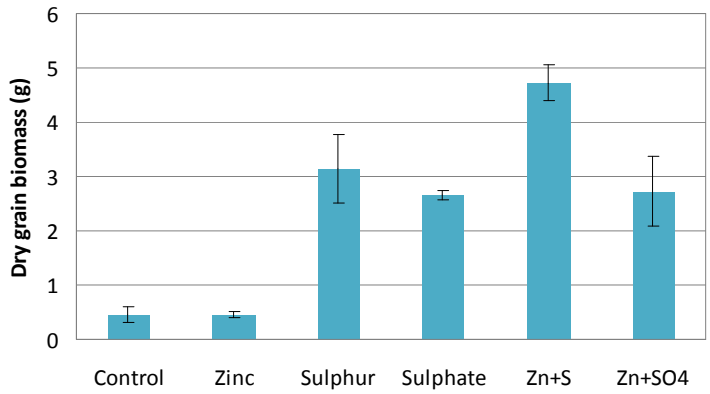



**Figure 25:** Total (a), shoot (b) and grain (c) dry biomass. Mean ± SE, N = 5 For grain biomass, the average of replicates that developed grains was taken.

In Figure 25 the mean biomass can be seen. Total biomass was influenced by the presence of any form of sulphur ($p<0.001$). This was also the case for the shoot biomass. Shoot biomass seemed higher if $SO_4$ rather than $S^0$ was added, but this was not significant. Grain biomass was significantly influenced by both sulphur ($p<0.001$) and zinc ($p<0.01$) additions. Both increased biomass, but $S^0$ additions increased grain biomass more than $SO_4$ additions ($p<0.01$).

The average total seed number per plant can be seen in Figure 26. Heads devoid of grains resulted in high standard errors. Sulphur ($p<0.01$) and Zinc ($p<0.05$) had a significant positive effect on the seed number. Although $S^0$ additions seemed to have a stronger effect than $SO_4$, this was not significant.

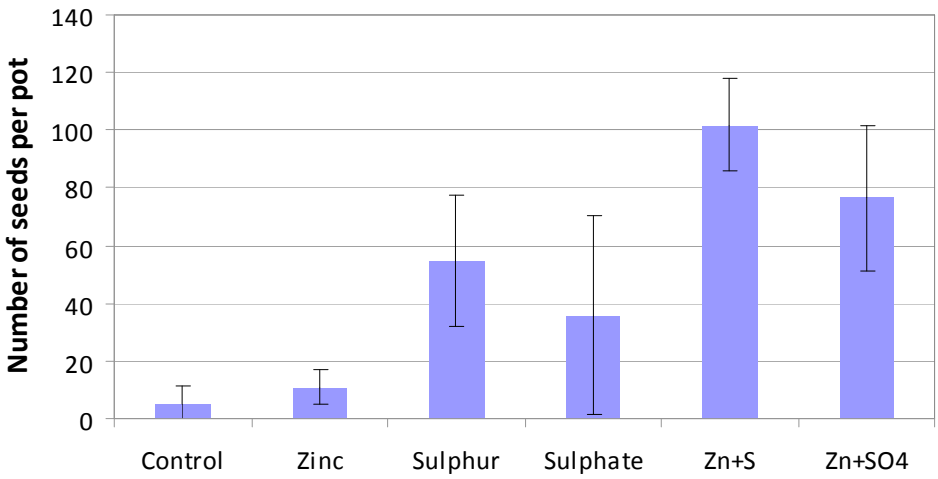



**Figure 26:** Number of seeds per pot (i.e. per plant). Mean ± SE, N = 5

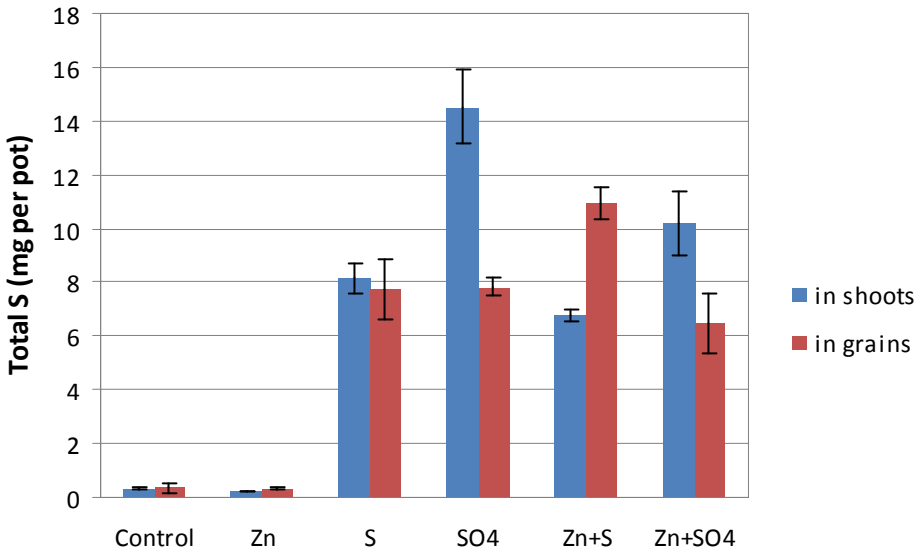
## 4.4 Plant element content

### *4.4.1 Total amounts of Zn and S per pot*

The total amount of S and Zn per plant can be seen in Figure 27. Total Zn content per plant in shoots was influenced neither by S nor by Zn additions. In contrast, total grain Zn content per plant was influenced by both S and Zn addition at a highly significant level ($p<0.001$). In each treatment having received Zn, grain Zn yields were higher than in the correspondent treatment without Zinc. Sulphur addition also increased total Zn in grain and $S^0$ was significantly highly than $SO_4$ ($p<0.05$).

Total S per pot significantly increased with the addition of both types of sulphur in grains ($p<0.001$), as well as in shoots ($p<0.001$). Shoot S yields were also significantly decreased by Zn addition ($p<0.01$), however no significant effect of Zn was observed in grains. $S^0$ and $SO_4$ treatments had a significantly different effect on grain and shoot S yields ($p<0.05$ and $p<0.001$ respectively): S yields were lower in grains but higher in shoots if $SO_4$ was added compared to $S^0$.

a)




b)

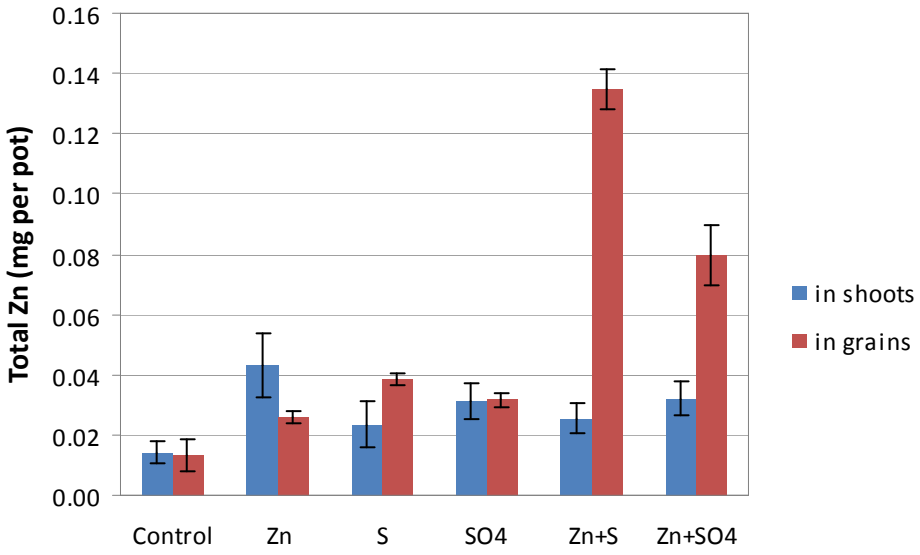



**Figure 27:** Total Zn content (a) and S content (b) in shoot and grains per pot. Mean ± SE, N = 5. For grain content, means of replicates that developed grains was taken.

### *4.4.2 Element concentrations*

*Zn concentrations*

S and Zn additions had a significant positive effect on Zn concentrations in wheat grains ($p<0.001$ in both cases (Figure 28a). While Zn inputs clearly increased grain Zn concentrations, $S^0$ and $SO_4$ inputs decreased Zn concentrations. No difference between $S^0$ and $SO_4$ treatments could be observed.

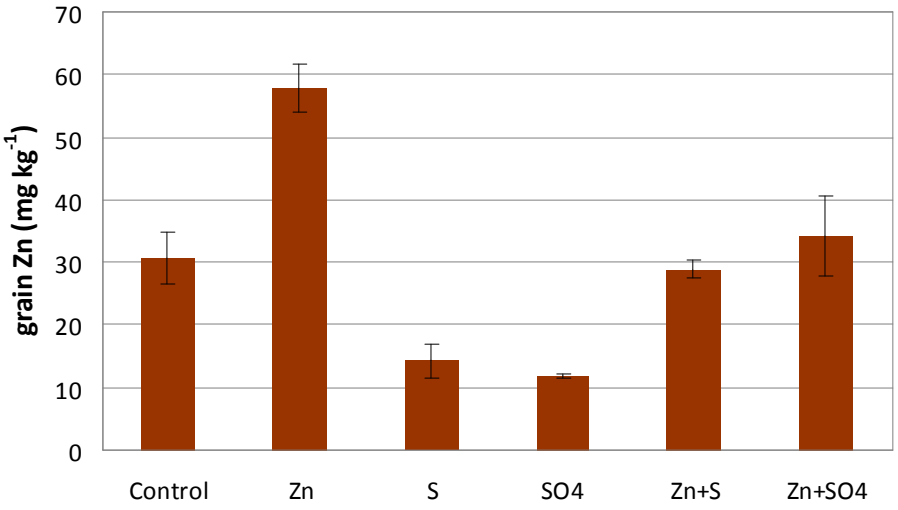
Zn concentrations in shoots (Figure 28b) follow the same trend as Zn concentrations in grains: Zn additions significantly increase concentrations ($p<0.001$) and S additions dramatically decrease the latter ($p<0.001$). A S*Zn interaction was found to be significant ($p<0.018$).
Since in the $SO_4$ treatment, three plants had heads with empty hulls, this part of the wheat spikes excluding the grains was analysed at the same time as wheat shoots. Zn concentrations of the hulls (cf. fig. b) looked very similar to the shoots of plants that were fertilised with $SO_4$.

*S concentrations*

S inputs had a significant effect on S concentrations in wheat grains and shoots ($p<0.001$, Figure 29): S concentrations were higher if any form of S was added. The slightly higher S concentrations found with $SO_4$ additions and the lower concentrations with Zn inputs that can be observed were not significant.

S concentrations in wheat shoots (Figure 29b) were significantly affected only by S additions, which increased S concentrations. Differences between $S^0$ and $SO_4$ were not significant.
S concentrations of the analysed hulls (cf. fig. b) were around 30% lower than the correspondent shoots, though.

a)




b)

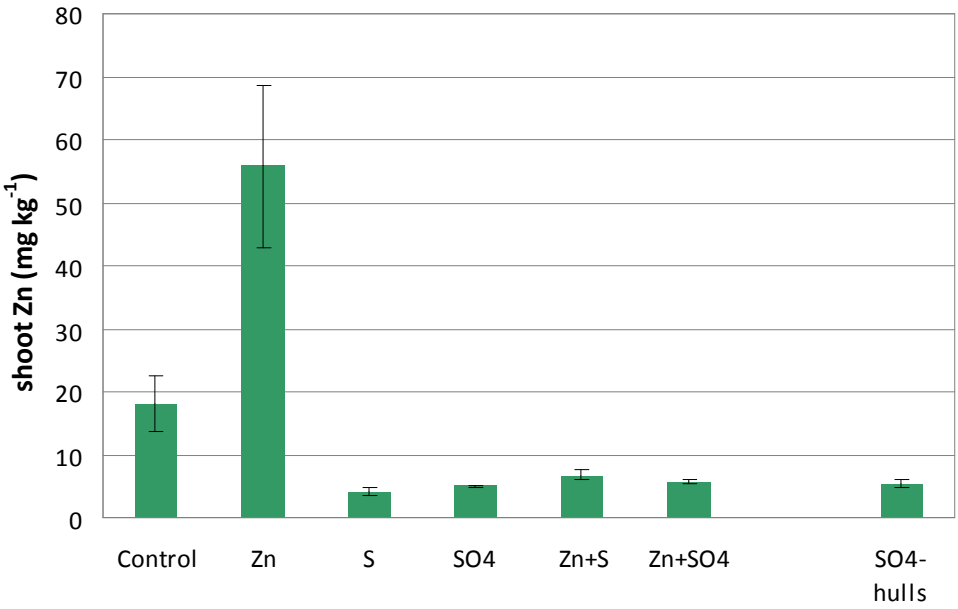
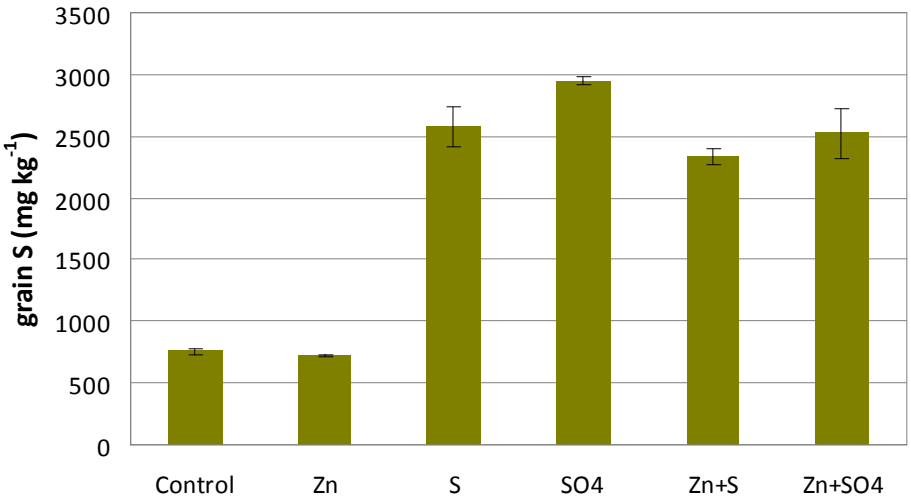



**Figure 28:** Zinc concentrations in wheat grains (a) and shoots (b) in mg $kg^{-1}$. Mean ± SE, N = 5 For grain concentrations, means of replicates that developed grains were taken.

a)




b)

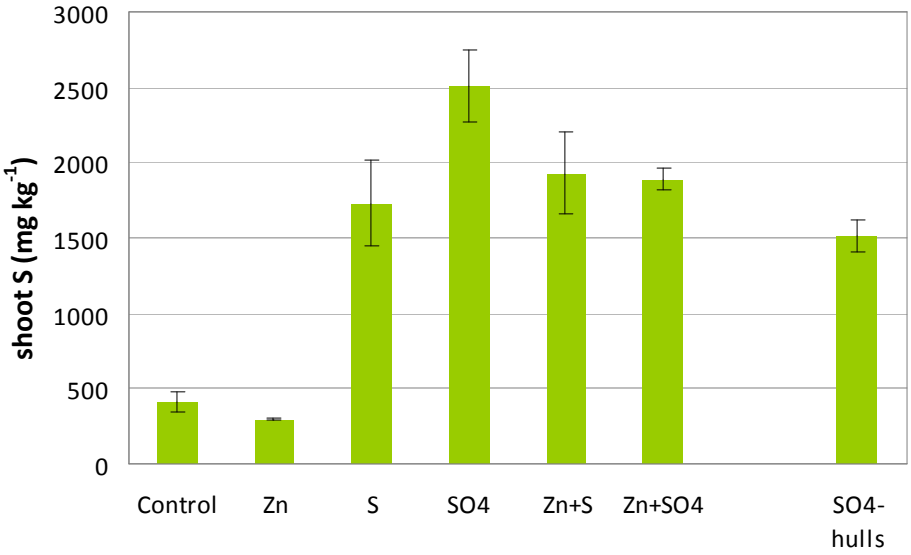



**Figure 29:** Sulphur concentrations in wheat grains (a) and shoots (b). Mean ± SE, N = 5. For grain concentrations, means of replicates that developed grains were taken.

*Manganese concentrations*

Figure 30 represents the manganese concentrations found in the grains. They follow the same trend as available soil Mn concentrations: sulphur affected this parameter significantly ($p<0.001$). Both treatments that had received $S^0$ had significantly higher Mn concentrations than all other treatments ($p<0.001$).

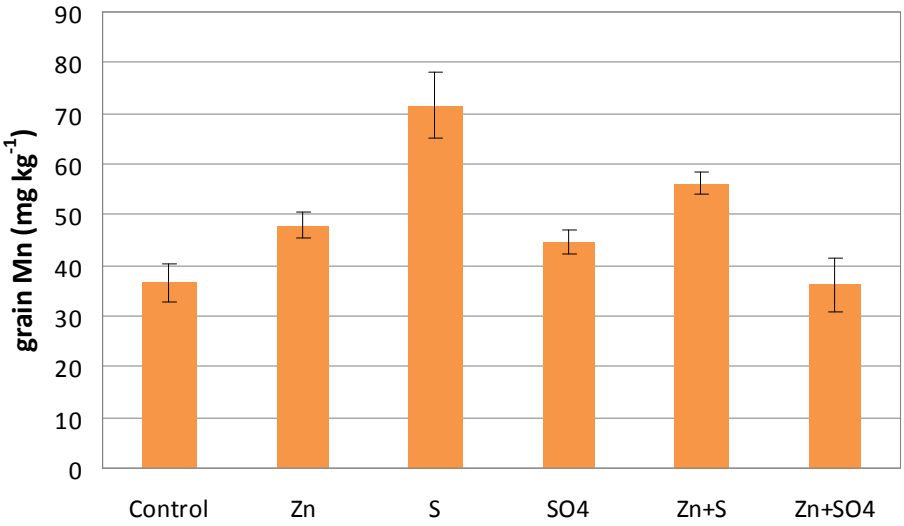



**Figure 30:** Manganese concentrations in wheat grains. Values of the replicates are means of the replicates that developed grains. Error bars represent standard errors.

*Plant concentrations of other elements*

Analysing treatment effects in shoot and grain Mg, K, Fe, Cu or Ca, was difficult because concentrations were often higher than the calibration range and some points may have been outliers. The data are shown in the appendix. In Figure 31 it can be seen that P grain concentrations were not influenced by any of the treatments. However, we will see that P concentrations are significantly correlated to many parameters.

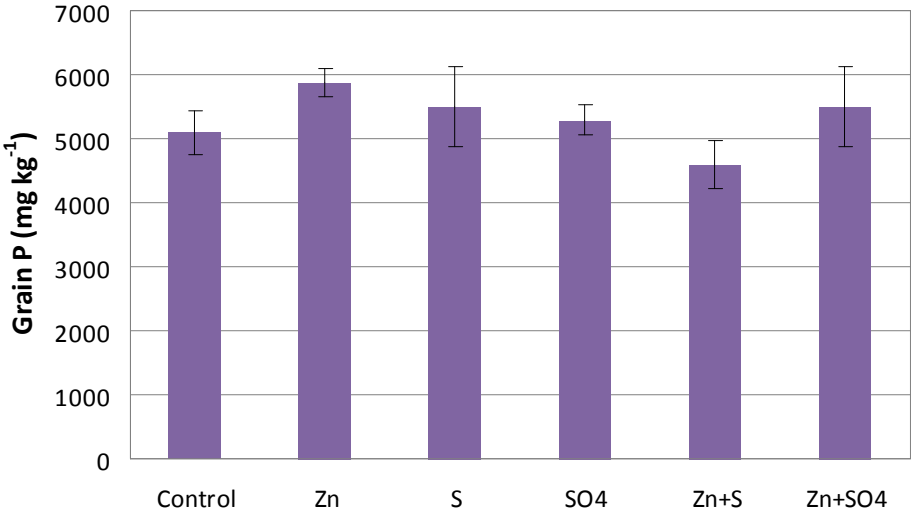



**Figure 31:** Phosphorus concentrations in wheat grains. Values are means of the replicates that developed grains. Error bars represent ± standard error.

### 4.5 Correlation Analysis

Correlation analysis between various grain, shoot, and soil parameters were undertaken. Table 10, Table 11, and Table 12 show the correlation coefficients ($R^2$). Several significant correlations were found.

As can be seen in Figure 32 a and b, there was a positive linear correlation between total zinc content per pot in grains and grain biomass ($R^2$ = 56.4%***), and an even stronger positive relationship between total S content per pot in grains and grain biomass ($R^2$ = 94.5%***). No clear correlation was found between grain biomass and Zn grain concentrations or S grain concentrations. Yet, if only treatments with Zn amendments are taken into account, Zn concentrations and biomass in grain show a highly significant negative correlation ($R^2$ = 77.0%***, Figure 33a).

We hypothesised that there would be a positive correlation between grain Zn and S concentrations due to the role of S in proteins and Zn's known association with protein in the grains (Morgounov et al., 2007; Broadley et al., 2007). Total grain Zn and total grain S contents per pot were indeed positively correlated ($R^2$ = 54.5%***, Table 10, Figure 34), while there was no clear trend between Zn and S concentrations ($R^2$ = 26.2%). However, if

only data points of those treatments that received both limiting nutrients, i.e. Zn and any form of sulphur, were plotted against each other, there is a highly significant positive linear relationship between grain Zn concentrations and grain S concentrations, according to our expectations ($R^2$ = 89.3%***, Figure 35).

**Table 10:** Coefficients of determination ($R^2$) between different grain and soil parameters. Only replicates with grains were taken into account.

| *Grains* | **Total Zn** | **Total S** | **Biomass** | **S conc.** | **Zn conc.** | **Mn conc.** | **Fe conc.** | **P conc.** |
|---|---|---|---|---|---|---|---|---|
| **Total S** (mg) | 0.544*** | | | | | | | |
| **Biomass** (g) | 0.563*** | 0.945*** | | | | | | |
| **S conc.** (mg $kg^{-1}$) | 0.128 | 0.402** | 0.214* | | | | | |
| **Zn conc.** (mg $kg^{-1}$) | 0.001 | 0.385** | 0.338** | 0.262* | | | | |
| **Mn conc.** (mg $kg^{-1}$) | 0.001 | 0.021 | 0.002 | 0.143 | 0.028 | | | |
| **Fe conc.** (mg $kg^{-1}$) | 0.113 | 0.051 | 0.024 | 0.089 | 0 | 0.205* | | |
| **P conc.** (mg $kg^{-1}$) | 0.137 | 0.300** | 0.469*** | 0.027 | 0.202* | 0.172 | 0.065 | |
| **Soil $SO_4$** (mg $kg^{-1}$) | 0.143 | 0.389** | 0.310** | 0.229* | 0.196* | 0.166 | 0.001 | 0.032 |
| **Soil Zn** (mg $kg^{-1}$) | 0.402** | 0.001 | 0.011 | 0.046 | 0.459** | 0.119 | 0 | 0.002 |
| **Soil Mn** (mg $kg^{-1}$) | 0.088 | 0.175 | 0.127 | 0.133 | 0.078 | 0.464*** | 0.062 | 0.002 |
| **Seed number** | 0.315** | 0.719*** | 0.814*** | 0.113 | 0.353** | 0.039 | 0 | 0.626*** |
| **Soil pH** | 0.453** | 0.394** | 0.342** | 0.241* | 0.003 | 0.032 | 0.03 | 0.025 |

* Significant at $P$ = 0.05; ** significant at $P$ = 0.01; *** significant at $P$ = 0.001

The correlation coefficients between total shoot S and total shoot Zn content per pot, concentrations of measured elements in shoots, shoot biomass and measured element's concentrations in soil are listed in Table 11. While, in shoots, total S content per pot is also significantly correlated to the shoot biomass (Figure 32 c, $R^2$ = 81.5%***), no specific trend could be observed between total Zn content in shoots and shoot biomass. As in grains, shoot S

and shoot Zn concentrations are not clearly correlated to shoot biomass. But if only treatments with Zn amendments are taken into account, Zn concentrations significantly decrease with increasing biomass in shoots ($R^2$ = 45.9%**, Figure 33 b).

**Table 11:** Coefficients of determination ($R^2$) between different shoot and soil parameters

| *Shoots* | **Total Zn** | **Total S** | **Biomass** | **S conc.** | **Zn conc.** | **Mn conc.** | **Fe conc.** | **P conc.** |
|---|---|---|---|---|---|---|---|---|
| **Total S** (mg) | 0.032 | | | | | | | |
| **Biomass** (g) | 0.1 | 0.815*** | | | | | | |
| **S conc.** (mg.kg$^{-1}$) | 0.029 | 0.613*** | 0.283** | | | | | |
| **Zn conc.** (mg.kg$^{-1}$) | 0.336** | 0.343** | 0.30** | 0.367*** | | | | |
| **Mn conc.** (mg.kg$^{-1}$) | 0.004 | 0.737*** | 0.648*** | 0.655*** | 0.557*** | | | |
| **Fe conc.** (mg.kg$^{-1}$) | 0.034 | 0.001 | 0.008 | 0.083 | 0.014 | 0.001 | | |
| **P conc.** (mg.kg$^{-1}$) | 0.084 | 0.431*** | 0.396*** | 0.503*** | 0.641*** | 0.745*** | 0.01 | |
| **Soil $SO_4$** (mg.kg$^{-1}$) | 0.079 | 0.09 | 0.076 | 0.262** | 0.165* | 0.199* | 0.057 | 0.274** |
| **Soil Zn** (mg.kg$^{-1}$) | 0.012 | 0.074 | 0.049 | 0.044 | 0.169* | 0.027 | 0.044 | 0.005 |
| **Soil Mn** (mg.kg$^{-1}$) | 0.009 | 0.011 | 0.075 | 0.03 | 0.022 | 0.05 | 0.061 | 0.099 |
| **Seed number** | 0.003 | 0.376*** | 0.259** | 0.339** | 0.143* | 0.333** | 0.036 | 0.276** |

* Significant at $P$ = 0.05; ** significant at $P$ = 0.01; *** significant at $P$ = 0.001

The distribution of the data in Figure 36, representing the Zn concentration in grains and in soils, shows higher grain Zn concentrations with higher available Zn concentrations in soil. The points are divided into two clusters of lower and higher concentrations and are significantly correlated ($R^2$ = 45.9%**). The cluster including low grain zinc and low available Zn in soil concentrations corresponds to the treatments that did not receive any zinc amendments, whereas the clusters with high grain Zn and high available Zn in soil contain the data points from treatments that received Zn. Nevertheless no clear trend relates grain S concentrations and soil available sulphate concentrations at harvest (Table 10). However this can be seen for Mn, there is a positive linear relation between Mn concentration in grain and available concentration in the soil ($R^2$ = 46.4%***, Figure 37).

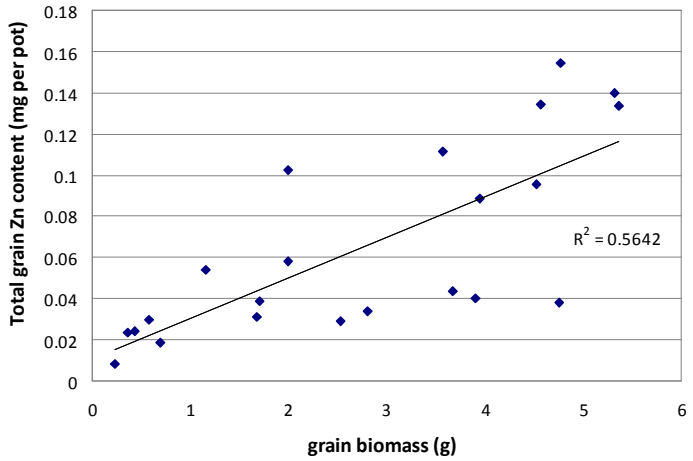
A highly significant negative linear relationship exists between the seed number and grain phosphorus concentration ($R^2$ = 62.6%***, Table 10) although there was no effect of Zn or S on this. With regards to biomass, total dry biomass was positively correlated to soil pH ($R^2$ = 36.6%***, Table 12, Figure 16). Also in plants that received any form of S amendments, grain biomass was negatively correlated to shoot biomass ($R^2$ = 75.9%*** Figure 38).

**Table 12:** Coefficients of determination ($R^2$) between soil parameters and total biomass

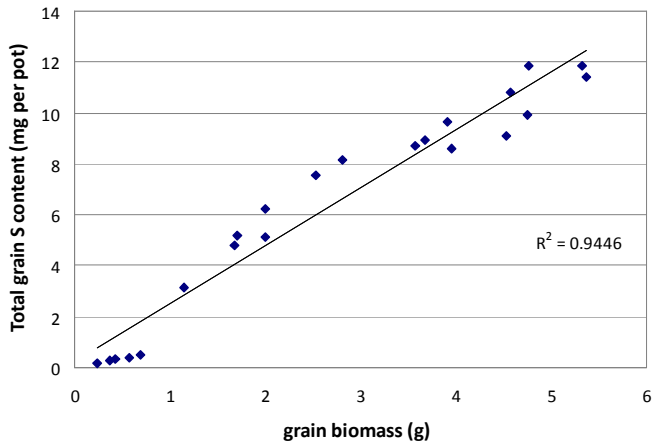
| *Soil* | **Avail. $SO_4$** ($mg.kg^{-1}$) | **Zn conc.** | **Mn conc.** | **Total dry biomass** (g) |
|---|---|---|---|---|
| **Zn conc.** ($mg.kg^{-1}$) | 0.009 | | | |
| **Mn conc.** ($mg.kg^{-1}$) | 0.488*** | 0.001 | | |
| **pH** | 0.114 | 0.046 | 0.004 | 0.366*** |

* Significant at $P$ = 0.05; ** significant at $P$ = 0.01; *** significant at $P$ = 0.001

a)




b)




c)

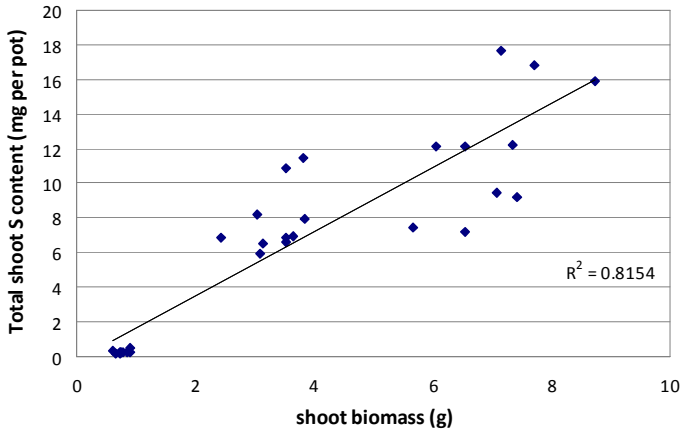
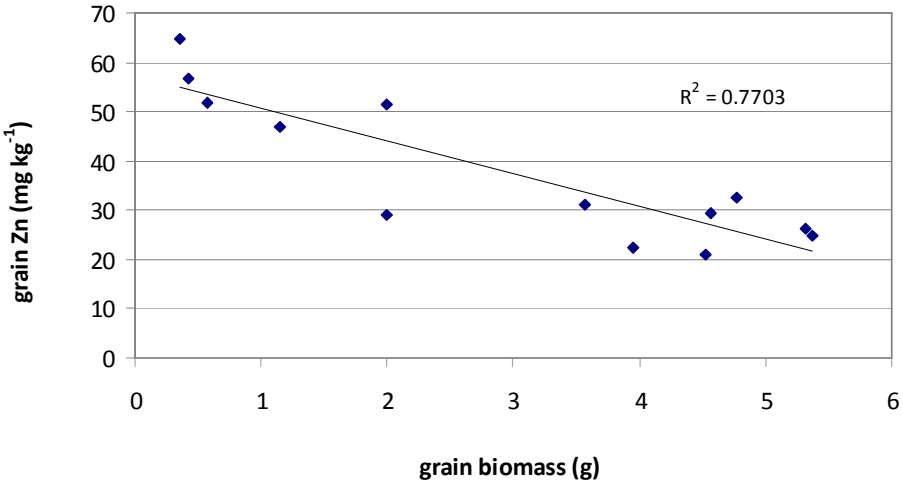



**Figure 32:** Linear regressions between total grain Zn content per pot (a) and total grain S content per pot (b) with dry grain biomass, and total shoot S content per pot with dry shoot biomass (c).

a)




b)

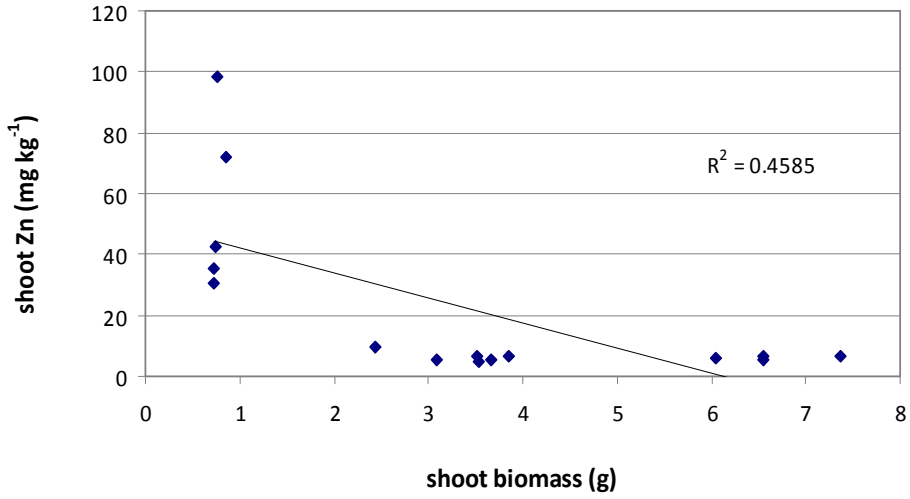



**Figure 33:** Linear regressions between grain Zn concentrations with grain dry biomass (a) and shoot Zn concentrations with shoot dry biomass (b). Only treatments that received Zinc are plotted.

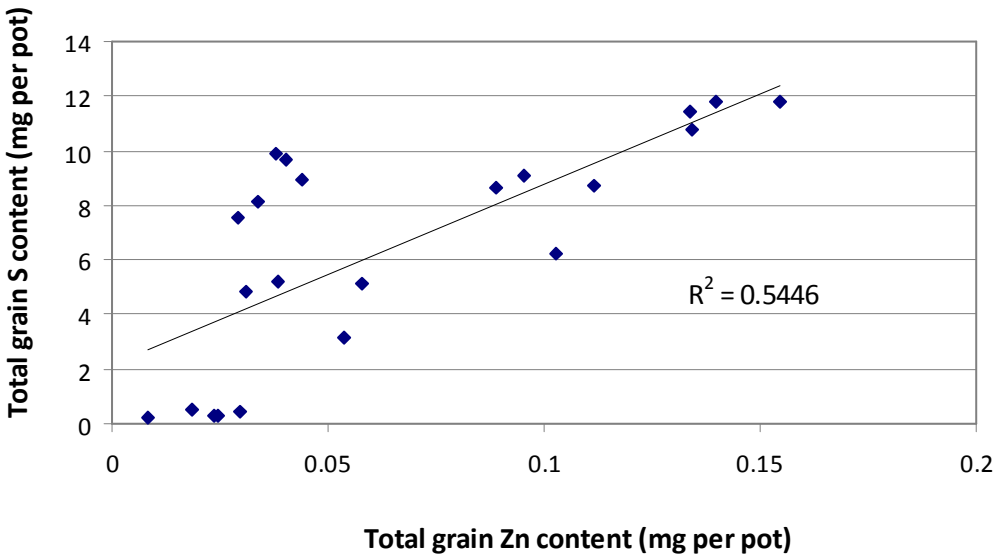



**Figure 34:** Linear regression between total grain S per pot and total grain Zn per pot

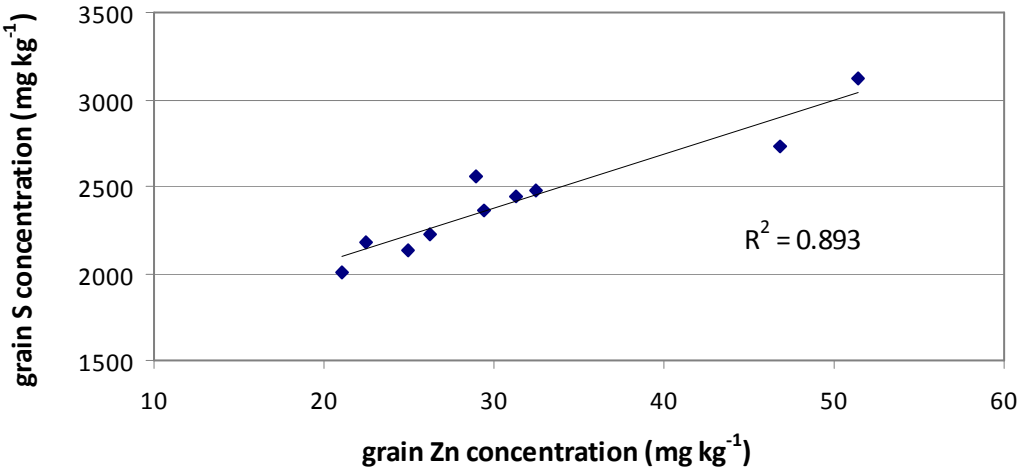



**Figure 35**: Grain S concentrations vs. grains Zn concentrations for treatments having received both S and Zn. i.e. the treatment $Zn+S^0$ and $Zn+SO_4$.

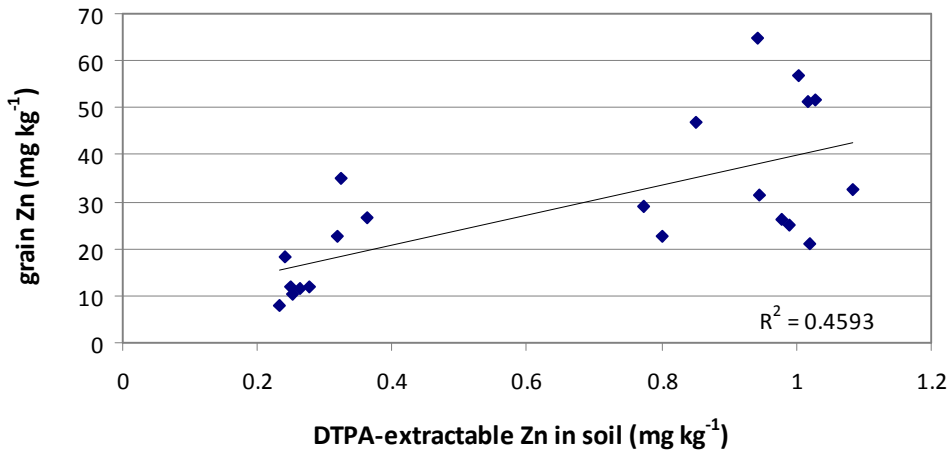



**Figure 36:** Linear regressions between Zn grain concentrations and soil Zn concentrations.

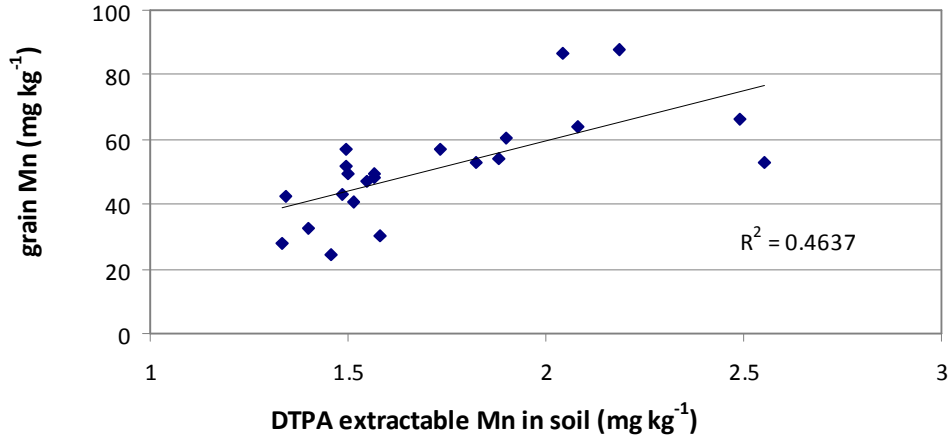



**Figure 37:** Linear regression between grain Mn concentrations and Mn concentrations in soil

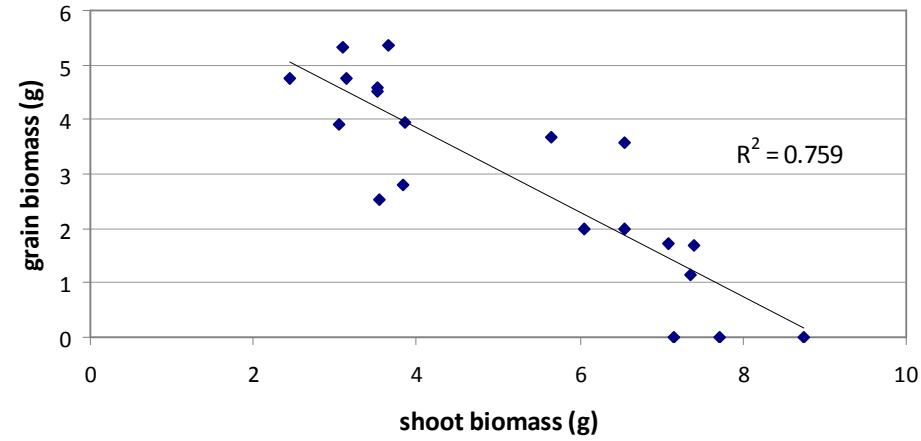



**Figure 38:** Linear regression between shoot biomass and grain biomass. Only plants that received sulphur are plotted. For plants that developed empty heads, the grain biomass is 0.

# 5 Discussion

## 5.1 Initial soil characteristics

The experimental substrate on which wheat plants were grown originated from the soil of an artificial catchment that had been in place for only a few years. This explains why it has low reactive surfaces areas for ion exchange and was initially deficient in almost every essential plant nutrient. These very unfertile initial conditions of the experimental substrate allowed us to interpret the results by excluding many parameters, such as the presence of organic matter that could have biased the results if "real" soil had been used. In addition, from its origin as underground Pleistocene sediment dumped on an artificial catchment (Gerwin et al., 2010), we know that the used substrate was not contaminated by any heavy metals or other anthropogenic pollutants. Additions of the fertiliser mixes at the beginning of the experiment provided the substrate with a sufficient level of nitrate, phosphate, calcium and potassium for plant growth, so that the potential limiting growth factors were sulphur, magnesium, copper, zinc, manganese, iron or boron. Although elements, such as magnesium might have been limiting, deficiency symptoms and growth patterns of plants could directly be related to sulphur and zinc, since Zn, $S^0$ and $SO_4$ were both added separately and in combination.

## 5.2 Soil analysis

A soil pH decrease was expected to take place in the $S^0$ and $Zn+S^0$ treatments, due to elemental sulphur oxidation, which, in turn, would increase DTPA-extractable Zn concentrations in soils and may, then, have increased Zn concentrations in wheat grains. However in this experiment the soil pH increased or did not change during the 17 weeks of the study. The buffering capacity of the soil seems not to have been exceeded despite the relatively low amount of calcium carbonate initially present (1%). Cui and Wang (2005) reported a pH decrease of 0.3 units after the addition of 100 mmol $S^0$ per kg soil, i.e. 3207 mg $S^0$ $kg^{-1}$ 54 days after the beginning of the experiment. Since our experiment was to compare the effect $S^0$ and $SO_4$ (as $K_2SO_4$) by adding the same amount of both sulphur forms, it was not possible to use such a high amount of $S^0$. Hence an $S^0$ input of 100 mg per kg soil was chosen, which is 32 times less than in Cui and Wang's study (2005). In planted pots the added $S^0$ amounts may thus have been too low to be able to notice any decreasing effect on pH. In

addition, while Cui and Wang (2005) added N as ammonium nitrate, we added only nitrate, which is known to increase the pH (Marschner, 1986)

Biomass production is another factor that could have caused the unexpected pH rise that particularly affected soil that received any form of sulphur. The pH was positively correlated to total biomass production ($R^2$ = 36.6%***). Pots that received any form of sulphur amendments had a significantly higher total biomass as well as a significantly higher pH compared to the control and Zn treatments. Biomass increase was the main visible effect that both forms of sulphur additions had on plant physiology. The correlation coefficient cited above is not very high because there were two clusters of the data points rather than a linear relationship between biomass and pH. One cluster was formed by pots with high biomass, i.e. plants that received any form of sulphur, and had a higher pH (around 7.9). The other cluster was constituted of the control and Zn treatments that developed only low biomass and had a lower pH (around 7.7). The mechanism responsible for this pH rise due to plant growth probably is the exchange in the rhizosphere between anions, such as sulphate and nitrate, taken up by the plants, and hydroxide ions ($OH^-$) or hydrogen carbonate ions ($HCO_3^-$) excreted by the plants, in order to maintain charge balance (Marschner, 1986).

The comparison between the pH of planted and unplanted soils also indicates that there was an effect of biomass production on pH. During the whole experiment, all unplanted soils had a lower pH than planted soils at harvest, with a differences of between 0.1 (control treatment) and 0.3 ($Zn+S^0$ treatment). The pH of unplanted soils showed a decrease of at least 0.5 units immediately after fertiliser additions, but, after approximately 30 days, it constantly increased with time until harvest, i.e. 120 days after the start of the experiment. In unplanted soils that received $SO_4$ amendments, more algae or fungi were visible at the surface and their soil pH was equal to unplanted control treatment at harvest, i.e. equal to the initial pH. Yet in the other unplanted pots (Zn, $S^0$ and $Zn+S^0$), there was a pH decrease of ca. 0.3 units compared to initial pH. Since the unplanted Zn pot had the lowest pH at harvest, we cannot say that $S^0$ caused the pH decrease. It may be due to soil formation with watering and fertiliser addition.

Cui and Wang's (2005) experiment with wheat lasted 54 days. Their initial soil pH was 7.23 in $CaCl_2$. Possibly, the pH of their soil could have risen (or decreased less) if the experimental time was longer. In that case the soil would have had time to buffer the initial reaction after

fertiliser addition and wheat plants would have had developed more biomass. This is even more probable if they only had used nitrate as a fertiliser.

However pH has been shown to decrease with $S^0$ additions also after a much longer time period. Over six years, Fässler et al. (2010) added more than 2100 kg $S^0$ per hectare per year to an experimental field, with an initial pH of 7.53, very close to the initial pH of 7.56 in our experiment. The annual added amount of $S^0$ corresponded, following very rough estimates, to a 2 fold higher amount than in our experiment. After 3 years of application, i.e. 6 times our $S^0$ application quantity, pH had reached a level that was 0.6 units lower than at the beginning. However, the authors added ammonium nitrate or ammonium as N fertiliser. Ammonium is known to decrease the soil pH, while nitrate can increase it (Marschner, 1986). This study was a field experiment, which means that plants had much more space to develop their roots. The sampled soil of Fässler et al. (2010) was thus less influenced by specific chemical reactions of the rhizosphere, in opposition to the pots of our experiment that were full of roots.

Root activity could, thus, have contributed to the observed pH increase during our experiment. Nevertheless, there could have been very localised pH decreases due to $S^0$ oxidation. In fact, significantly higher concentrations of DTPA extractable Mn was found in treatments that received $S^0$ amendments. However Mn is only reduced and thus mobilised in acid conditions (Marschner, 1986). Mn reduction requires an electron donor that $S^0$ oxidation, i.e. acidity production could provide. It is possible that the increase in pH mainly occurred in regions of the soil that were close to roots, while pH decreases happened locally in $S^0$ and $Zn+S^0$ treatments. Moreover, since the fertiliser was mixed manually, a totally homogeneous $S^0$ content was not guaranteed, so the pH may have locally decreased and led to Mn oxidation because of the presence of $S^0$ aggregates in the soil.

Available sulphate concentrations slowly increased over time in soils of the $S^0$ and $Zn+S^0$ treatments. This confirms the slow sulphate release capacity of $S^0$, which is a prerequisite for our hypothesis that maturity and Zn remobilisation is favoured due to sufficient $SO_4$ concentrations during grain-filling. Virtually no available sulphate was present after harvest in soils of control and Zn treatments. However about 1.5 to 2.25 mg $SO_4$ per pot were taken up by the above-ground part of the wheat plants. The initially very low available sulphate amount present in the substrate, 7.2 mg $SO_4$ $kg^{-1}$, must have been used as a S source. In $SO_4$ and $Zn+SO_4$ treatments, available soil sulphate concentrations at harvest were in the range of

the critical concentration for plant growth (24 to 30 mg $SO_4$ $kg^{-1}$, Scott, 1981), whereas in the $S^0$ treatments, a two to three times higher available soil sulphate concentration was measured in the soil compared to $SO_4$ treatments. This could be due to the fact that at the beginning of the experiment, $SO_4$ was readily available to plants in the sulphate treatments, whereas $S^0$ first needed to be oxidised to $SO_4$ over time. This slow release meant it could not be completely taken up by biomass from the start of the experiment. This is in accordance with our initial hypothesis.

In the $S^0$ treatments about 75% of the 100 mg added $S^0$ probably was, once oxidised into sulphate, consumed by biological activity, while in the sulphate treatments, about 90% of the relatively high amount of added $SO_4$-S (100 mg per kg soil) was used by biological activity during the experiment. In all treatments that received sulphur, the above-ground part of the wheat plants took up between about 15 and 23 mg S per pot. Thus, even considering the sulphur contained in roots, the main sulphur source for wheat plants should have been the 100 mg S added as fertilisers and no sulphur had to be mobilised from the soil. The $S^0$ amendment in this experiment was already sufficient to obtain adequate soil sulphur concentrations for wheat growth.

Soils of the control, $S^0$ and $SO_4$ treatments were clearly deficient in available Zn at harvest, with less than 0.4 mg Zn $kg^{-1}$ (Alloway, 2009). Since their above-ground biomass never contained more than 0.1 mg Zn per pot, plants probably used up the initially present 0.19 mg $kg^{-1}$ available Zn in the soil without being able to mobilise the 16 mg $kg^{-1}$ total zinc measured in the substrate. DTPA extractable Zn concentrations were significantly higher in soils where Zn fertiliser was added, which is in accordance with Moraghan et al. (1999). In contradiction with our hypothesis, available Zn concentrations were not influenced by sulphur additions, probably because of the lack of expected pH drop described above or because.

Available Zn concentrations in soil remained unchanged with time. This is in accordance with the absence of a decrease in soil pH over time, which was expected to enhance the available soil Zn pool. At harvest, available Zn concentrations were situated in the critical to adequate concentration range for wheat growth of about 1 mg available Zn per kg dry soil. Not more than 10% of the 2 mg added Zn was utilised by above-ground biomass. We thus consider that, for adequate growth, the wheat plants, roots included, did not need to mobilise Zn initially present in the soil because added Zn was still left. But they also may not have been able to

mobilise Zn because soil Zn, originating from the soil or from the fertiliser, was too strongly bound to soil particles. However, even if the available Zn concentrations are considered sufficient for plant growth, increasing them to adequate or high available levels may lead to higher Zn concentrations in grains (Moraghan et al., 1999; Wang et al., 2011). In fact, in contrast to $SO_4^{2-}$, $Zn^{2+}$ is a relatively immobile ion in soils (Jones and Jacobsen, 2001), so it is possible that the higher the available Zn, the more Zn will be taken up by plants.

### 5.3 Wheat growth and biomass

Total dry biomass was primarily influenced by sulphur additions, since S is an essential plant macronutrient and the soil was initially sulphur deficient. Differences in biomass between $S^0$ and $SO_4$ treatments were observed when grain biomass was distinguished from shoot biomass. In fact elemental sulphur had a significant positive effect on grain biomass and shoot biomass significantly decreased when grain biomass increased. A probable explanation for this phenomenon is that in the $Zn+S^0$ treatment, as sulphate was present in smaller amounts at the beginning of plant growth, but was still released during the generative growth phase, the wheat heads could develop better. In the $Zn+SO_4$ treatment, where sulphate was readily available at the beginning, large amounts of sulphate could have been taken up during vegetative growth, leaving the sulphate supply too low for adequate generative growth. This would confirm our initial hypothesis that elemental sulphur additions have more effect on wheat grain yields than sulphate because sulphate release into the soil solution is slower so that there is still sufficient sulphate available during the grain-filling phase. In fact, Yang and Zhang (2006) stated that unfavourably delayed senescence results in a low grain-filling rate. Since under S deficiency, active enzyme synthesis is given the priority at the expense of S containing storage proteins (Zörb et al., 2010), S nutritional status may be crucial for both grain yields and grain quality. This is supported by Eriksen and Mortensen (2002) who highlight the importance of availability of soil S during grain filling for adequate grain protein quality in barley.

The expected beneficial effect of slow sulphate release due to elemental sulphur oxidation was already observed in the different growth patterns and deficiency symptoms that varied with the treatment. Wheat plants of the control and Zn treatments grew very similarly: they had few small and light coloured leaves, poor tillering and poor heading. Since N, P and K were present in sufficient amounts, these observed symptoms must have corresponded to S

deficiency (Bergmann, 1992). However potential S deficiency could also be observed in both $SO_4$ treatments when generative growth started: most of the wheat heads only appeared during the second growth phase, not all wheat ears reached anthesis, and at harvest, many plants were still partially green, whereas the $S^0$ treatments generally headed earlier and all wheat ears reached anthesis. These observations correspond to the S deficiency symptoms "retarded growth, older leaves remaining green, reduced flowering and reduced grain filling" described by Tandon (2011).

Zn deficiency symptoms are known to be difficult to distinguish from other symptoms (Bergmann, 1992). Many symptoms such as light green leaves or few fruits could also be attributed to S deficiency. Chlorosis was never observed in this experiment and the necrotic patches in the $SO_4$ treatment seemed too dark and uniformly distributed to be explained by Zn deficiency. Moraghan et al. (1999) reported that adequate Zn fertilisation hastened maturity of heads. In accordance with the latter study, in our experiment, Zn, $Zn+S^0$ and $Zn+SO_4$ treatments reached anthesis earlier than control, $S^0$ and $SO_4$ treatments respectively. Also, $Zn+S^0$ and $Zn+SO_4$ treatments had a higher number of wheat ears than $S^0$ and $SO_4$. Although control and Zn treatments developed the same number of heads, the latest control wheat ear was empty. So grain development may have been promoted by Zn additions.

Altogether, every treatment except $S^0$ and $Zn+S^0$ was affected by S deficiency, but not at the same growth phase: control and Zn treatments from the tillering stage, and $SO_4$ and $Zn+SO_4$ during generative growth. S and Zn sufficiency had similar effects that could be aggregated. They both hastened maturity and promoted higher head number. In addition to a higher grain production, from all treatments that received any form of sulphur, $Zn+S^0$ fertilised plants were the only ones that did not developed any mutant wheat heads. They seem to have had the "healthiest" developed in this experiment.

### 5.4 Plant element contents

Both tested factors S and Zn had no influence on total Zn content in shoots for all treatments, whereas in grains, total Zn contents were more than twice as high in the $Zn+SO_4$ compared to the $SO_4$ treatment, and at least 3 times higher in the $Zn+S^0$ compared to the $S^0$ treatment. In contrast, total S content in shoots notably increased with sulphur additions, it was higher

when $SO_4$ rather than $S^0$ was added, and it decreased with zinc additions. S content in grains was highest in the Zn+$S^0$ treatment.

The results show that, in accordance with our hypothesis, the slow sulphate release characteristic of elemental sulphur may have influenced the nutrient distribution between generative and vegetative plant parts. Sulphur, which has a low mobility in plants (Jones and Jacobsen, 2001) was mainly contained in shoots of the plants treated with sulphate. Wheat may not have been able to remobilise S from its own biomass for grain storage when the easily available sulphate in soils became depleted. This could mean that the sulphate treatments took up a lot of sulphur from the soil before generative growth started, and S was no longer available afterwards. In an analogous way, the S uptake of the $S^0$ treatments may have been more moderate in shoots and higher in grains compared to all other treatments because enough sulphate was still available in soils when wheat head formation started.

Further, our findings also suggest that Zn is remobilised from old leaves into the grains once the shoots have sufficient zinc. Zn is considered to have a relatively low mobility in plants (Jones and Jacobsen, 2001). Nevertheless it is more mobile than other trace elements such as Mn or Fe in wheat (Riesen and Feller, 2005), and Zn has been shown to be easily translocated through the phloem from the vegetative tissues, especially from the senescing flag leaves, towards the grains (Erenoglu et al., 2011; Moraghan et al., 1999). In our study, for shoots and for grains, a minimum Zn level between 0.02 and 0.04 mg Zn per plant seems to have been needed for growth. Once this level was reached, the remaining Zn could have been stored into the grains, probably when senescence of the leaves, especially the flag leaves, started. This would explain the absence of positive correlation between total shoot Zn content and shoot biomass. Zn translocation from leaves to grains is also supported by the fact that plants that developed faster, i.e. plants whose leaves started senescence earlier and had reached physiological maturity at harvest, had generally higher total grain Zn contents than plants that developed wheat ears in a second phase around the $10^{th}$ week of the experiment. So the previously described effect of $S^0$ enhancing wheat maturity, rather than the proposed pH effect of $S^0$ on soil Zn availability explains the high total Zn contents in the $S^0$+Zn treatment.

One reason why enhanced maturity may trigger Zn translocation to the wheat grains is that the S nutritional status of wheat might have a major effect on Zn transport to the grains in a similar way that N supply was proposed to affect Zn transport. Erenoglu et al. (2011)

suggested that increasing N concentrations in tissue may enhance root Zn uptake, xylem and phloem transporter proteins. Since Zn has a high affinity binding to S functional groups (Regvar et al., 2011), the potential role of S in transport proteins should be taken into consideration. However a high available Zn status may also be responsible for the increase of Zn transport in plants (Erenoglu et al., 2011), as well as for a higher sulphate transport in plants (Na and Salt, 2011).

Zn and S concentrations in wheat hulls were measured separately in the $SO_4$ treatment because three out of five plants did not develop any grains. Zn concentrations of hulls were notably closer to shoot than grain concentrations. So Zn was, indeed, transported into the grains themselves instead of the outer parts of wheat ears. However in all five $SO_4$-replicates, S concentrations of the hulls were lower than in shoots, and shoot concentrations were lower than in grains. This indicates that the proportion of S, which reached the heads despite its low remobilisation was transported in priority to the grains. Low grain biomass in the sulphate treatments may explain why S concentration in grains are as high as in the $S^0$ treatment. S probably went into the S containing grain proteins, since the other main constituents of seeds are carbohydrate and oils (Egli, 1998).

Moreover, the comparatively high total grain Zn and S contents of the Zn+$S^0$ treatment of our study, as well as the significantly positive correlation between total Zn and total S content in grains, indicate that Zn transfer and accumulation in the grains may have been linked to the higher S amount in grains. This would confirm our hypothesis that the Zn grain content increases with S grain content due to the grain protein's role as a Zn sink (Erenoglu et al., 2011, Kutman et al., 2010), e.g. in storage metalloproteins (Alloway, 2008), or as a peptide ligand on structural or catalytic enzyme sites (Persson et al., 2009, Broadley et al., 2007).

Positive correlations between grain Zn and grain protein have been shown in various studies (Morgounov et al., 2007; Cakmak et al., 2004). However the significant positive correlation found in this study between total Zn and total S content in grains is not sufficient to show the linking role of protein sulphur for Zn accumulation in grains. If Zn is stored in grain proteins (Kutman et al., 2010) and is involved in enzyme metabolism (Broadley et al., 2007), grain Zn concentrations and grain S concentration should be positively correlated. This is not the case at first sight ($R^2$ =26.2%*). However the initial substrate was very deficient in Zn and S. So these limiting factors may have biased the results. On one hand, when all treatments were

considered, we found significant positive correlations between total Zn content in grains with grain biomass. On the other hand, Zn concentrations in grains and shoots were negatively correlated with grain and shoot biomass respectively if only treatments that received Zn were considered. This indicates that there was a dilution effect for Zn concentrations in grains and shoots. Yet, if only those treatments that presented no limiting factor, i.e. $Zn+S^0$ and $Zn+SO_4$, are plotted, grain Zn concentrations and grain S concentrations are, indeed, very significantly positively correlated. This further supports our hypothesis that sulphur in grains, which is mainly contained in storage proteins (Egli, 1998; Bergmann, 1992, Zörb et al., 2010), helps to accumulate Zn in an analogous way to that of N has been shown to do (Kutman et al., 2010).

These correlations and dilution effects were also described in the study of Singh et al (1986) who found that total Zn and S contents in wheat grains were positively correlated, but Zn concentrations decreased with $S^0$ application, which they explained by a higher biomass production if sulphur was added. They deduced a critical S/Zn ratio of 103.7 for wheat grains.

In grains and shoots respectively, S concentrations were generally similar between treatments that received S inputs. Without being influenced by the form of added S, S concentrations were higher than the critical level of 0.12% in grains given by Reuter and Robinson (1986) in all treatment but control and Zn, which were obviously sulphur deficient. Grain S concentrations were always higher than shoot S concentrations because the plant's proteins accumulate in the grains with senescence (Eriksen and Mortensen, 2002).

According to Reuter and Robinson (1986) the critical grain Zn concentration for wheat growth is in the range of 5 to 10 mg $kg^{-1}$, whereas Alloway (2009) considers a concentration of 15mg.$kg^{-1}$ in grains and 18 or 20 mg $kg^{-1}$ in shoots as critical for plant growth. These values suggest that grain Zn concentrations in the $S^0$ and $SO_4$ treatment were low or critical at harvest, whereas all other treatments, including the control and Zn treatment, had a sufficient grain Zn concentration. In shoots, the Zn treatment was the only one whose concentration clearly was above the critical level.

Depending on their genotype and the ploidy of wheat varieties, Zn concentration in wheat grains may reach 180 mg $kg^{-1}$ (Cakmak et al., 2000). Gomez-Becerra et al. (2010) termed a grain Zn concentration of 70 mg $kg^{-1}$ and grain protein concentration of 30% as high. The comparably very high Zn concentrations in shoots and grains of the Zn treatment (almost 60

$mg.kg^{-1}$ in both cases) that can be explained by lack of dilution by biomass indicate how much higher the Zn concentrations could have been if more Zn would have been available in the soil per biomass unit. In fact grain Zn concentrations and available Zn concentrations in soil were positively correlated. Thus, although in this experiment the added amounts of $S^0$ fertiliser had a beneficial effect on grain Zn concentrations compared to a single sulphate addition, adding more $S^0$ in order to obtain a pH decrease with the subsequent available Zn increase would probably have further helped to achieve the goal of biofortification, i.e. high Zn concentration in wheat products.

Available soil manganese concentrations were significantly increased by elemental sulphur. This could have been due to local acidification originating from $S^0$ oxidation. Since Mn is also an important plant micronutrient (Marschner, 1986), the positive correlation between soil Mn and grain Mn concentrations highlights a possible beneficial effect of elemental sulphur compared to sulphate fertilisation that does not require higher sulphur fertiliser application rates in soil than in this study. Kutman et al. (2010) found that wheat grain concentrations of Mn, but also Fe and Cu responded positively to increasing N supply. They attributed this trend to possible similar N-dependent mechanisms as Zn for translocation or storage into the grains. Cu and Fe concentrations measured in our experiment, although not very accurate and not always significant (data not shown), also indicated a positive effect of $S^0$ on element grain concentrations.

The largely concordant results between our study and Kutman et al. (2010) - where N and Zn fertilisation had a synergistic effect on grain protein Zn concentrations - reinforce the validity of the statement that adequate S and Zn fertilisation may induce greater Zn accumulation in grains due to their role in protein metabolism. Overall, sulphur and nitrogen may both play an important role in trace element biofortification of wheat grains due to their presence in proteins, but probably in different ways. According to Zörb and Grover (2010), N could affect protein concentration and biomass production to a greater extent, while S would affect storage protein composition.

In this experiment we did not differentiate between different parts of the wheat grains with regards to Zn allocation. Yet, while mainly the endosperm, i.e. white flour is consumed by people, the outer part of the grain, i.e. embryo and aleurone contain substantially higher Zn concentrations. According to Kutman et al. (2011), increased N supply positively affects Zn

concentrations of all parts of the wheat grain, with a more pronounced effect on the endosperm fraction. The endosperm contains also markedly lower phytate concentrations than the outer parts of the grains, which reinforces the idea that grain proteins are more important Zn sinks than phytic acid (Kutman et al., 2011). Persson et al. (2009) showed that Zn in wheat was mainly bound to peptides, while Fe was mainly associated with phytic acid. In our study no significant correlations were found between grain phosphorus concentration on one side and grain Zn, S, and Fe concentrations respectively on the other side, while it was significantly correlated with grain biomass and the number of seeds. This also strengthens the idea of S supply increasing Zn content specifically in grain proteins.

# 6 Conclusion

In this study, $S^0$ and $SO_4$ had different effects on wheat yields and grain Zn content. All but one hypothesis were verified. In planted pots, contrary to expectations, no pH decrease was observed after $S^0$ additions as it had been found in other studies, but the pH slightly increased with plant biomass production. This could be explained by several factors: (i) the soil buffering capacity was not exceeded, (ii) the soil was fertilised with nitrate, which increased pH, and (iii) biomass production implied anion uptake, which was balanced by exudation $OH^-$ and bicarbonate ions. However the occurrence of local acidification was implied by an increase in available Mn concentration in soil and in grains if $S^0$ was added.

With regards to biofortification, Zn and $S^0$ additions, separately and in combination, resulted in Zn grain concentrations that were equal or higher than the critical available level for plant growth. However the positive correlation between grain Zn and soil Zn suggested that Zn in grain could reach even higher concentrations if more Zn was available in soils. A large part of the added Zn was probably adsorbed by soil particles and the original total soil Zn content seemed to be bound too strongly to be mobilised. A bigger pool of soil available Zn could thus induce higher grain Zn concentrations in wheat grains.

According to our hypothesis, Zn and S total content in grains were significantly positively correlated. It was also the case for Zn and S concentrations if the dilution effect in the control and Zn treatments was not considered. This suggests that Zn is bound in S-containing grain proteins and thus Zn storage in grain is enhanced, which is similar to the results of the studies that associated proteins and Zn by looking at N. It also dismisses the theory that Zn is mainly stored in phytate complexes in grains. No correlation was found between grain zinc and phosphorus that could have indicated that Zn storage with phytic acid was occuring in our experiment.

The significant positive relationship between Zn and S in grains could be synergistic. In general, Zn cations and reactive S groups have a high affinity. Also, on the one hand, proteins store and transport Zn, while on the other Zn protects the plant against reactive $O_2$ species (this is important for photosynthesis, i.e. building the plant before grain filling) and protein synthesis is depressed by Zn deficiency due to its role in RNA and ribosomes.

$S^0$ released $SO_4$ slowly as expected. This had many beneficial effects on wheat grain and Zn yields. In fact sufficient sulphate was available for grain filling, which lead to comparably high wheat grain yields at harvest. More total Zn and total S reached the grains if $S^0$ was added, and particularly with both Zn and $S^0$ additions. S and Zn additions may have allowed Zn remobilisation from leaves to grains because it accelerated maturity. In contrast, in the sulphate treatments plants developed very fast when $SO_4$ was readily available meaning available sulphate soil concentrations at harvest were not higher than the critical level for growth. So wheat plants did not have enough S supply to fully reach maturity, resulting in less grain filling and less total Zn.

Overall it is very likely that Zn and S nutritional status are directly – via their roles in plant metabolism - and indirectly - through an effect of nutrient mobilisation and delayed maturity - linked with regards to protein composition, Zn transport, and storage. The effects could be more specific than the effects of N, because only cysteine and methionine, which are of major importance because of their binding functions, contain S in proteins.

# 7 Outlook

Zn deficiency is a major health problem if a diet mainly consists of staple-food, such as wheat products, which is the case for the majority of the people in poor regions of the world. Because Zn deficient, often alkaline, soils cover large arable areas, Zn deficiency also affects plants and has major detrimental effects on their development. Meanwhile, sulphur deficiency in agricultural soils is becoming a concern in industrialised countries because air pollution, S use as a pesticide and S impurities in fertilisers are decreasing. In developing countries where fertilisation is comparably expensive, macronutrients are soon exhausted in cultivated soils. The nutritional status of Zn and S in wheat may be tightly linked. In fact S is a major component of proteins, which have been shown to be associated with Zn storage in wheat grains in studies on nitrogen or on proteins in general.

As far as we know, the relationship between protein S in cereal grains and grain Zn content has not been studied satisfactorily. Sulphur applications to soil have shown positive effects on Zn biofortification in wheat grains. This was mainly related to pH decreases that enhanced Zn bioavailabilty in soils. But since sulphur is an essential macronutrient, it was important to look at different S fertilisation forms in order to determine if Zn increased due to higher availability in soils or facilitated storage in wheat grain proteins. This could allow identification of the best type of fertiliser for different soil types. While research has focussed on Zn complexation with phytate, a lack of knowledge exists about Zn storage in grain proteins. Although Zn and –SH groups are known to bind with high affinity, the relationship between Zn and S transport through rhizosphere, xylem, and phloem into the grains needs further investigation. In addition this could help determine if fertiliser amendments to the soil are more or less efficient than foliar sprays. With regards to Zn transporters, research has also focussed on N, while S may have a large influence.

This study showed that, from the point of view of biofortification, adequate Zn nutritional status in the plant and sufficient S availability at the grain-filling stage was crucial to obtain high grain Zn concentrations in wheat plants that developed high biomass because both elements may be linked in transport and storage processes. However sulphur was only sufficiently available at harvest if $S^0$ rather than $SO_4$ was added and Zn was only available if it had been added. The different impact of fertilisation on Zn biofortification depending on the

growth stage highlights the importance of conducting studies that last until wheat harvest, in order to have a complete overview of the effects of the treatments over time. Eventually the focus has to lie on the Zn content of the final product, i.e. wheat grains. Moreover, the preparation of meals out of flour may also substantially influence Zn availability. Especially the fate of phytate during meal preparation and digestion is relevant since it influences Zn bioavailability in humans. Besides, mainly the endosperm, i.e. white flour is used for consumption, although wheat bran (aleurone) contains much higher Zn concentrations. In the context of biofortification, studies should take into account food habits of the consumers in order to focus on the Zn concentration in specific parts of the grains.

Zn is not the only major deficient micronutrient in humans. Iron deficiency is also a main health risk attributed to food intake. However Zn and Fe are both two divalent cations and are known to interact in the metabolism of plant and human organisms. Biofortification of Zn should not induce Fe deficiency. Research on the effect of S fertilisation should thus be extended to both interacting elements in order to avoid trade-offs between the two.

From a farmer's point of view, the combination of $S^0$ and Zn fertiliser showed the best results because wheat grain yields were the highest and wheat growth was the fastest compared to treatments without Zn or with sulphate amendments. In the field, the mobile $SO_4$ is easily leached with rain or irrigation water, whereas $S^0$ is only mobile after it has been oxidised. Higher application rates than those in this study could have resulted in a pH decrease and subsequently higher Zn availability. However N fertilisation with urea could be another option to acidify the soil. In alkaline soils, Zn is easily adsorbed. So soil acidification should be combined with Zn fertilisation to ensure sufficient Zn supply

In acid soils, acidification may lead to heavy metal toxicity. So if acid soils are S deficient, sulphate additions are more appropriate than $S^0$. When S is added directly as $SO_4$, the application time and rate is very important. Too much $SO_4$ might delay senescence and the moisture of the soil should be considered to avoid leaching. In addition to S amendments at the start of wheat growth, S fertilisation after anthesis, analogue to late N fertilisation seems to be a good application time. The effect of application time of $SO_4$ could be seen in our experiment due to slow release from $S^0$. However the experimental design did not allow us to observe effects on Zn application time, which may be as important as the application time of S and should hence be further explored.

Overall, Zn and S fertilisation in adequate amounts seems very useful for agriculture in general and for biofortification of Zn in wheat in particular. The fertiliser amounts have to be put in context with other fertilisers, such as N or P. It should not be forgotten that ratios such as S/Zn or N/S in plants are as important as the absolute quantities of the element added to the soil in order to detect nutrient deficiencies.

In the future, Zn and S deficiency, as well as other nutrient deficiency may be reduced by plant breeding. This requires a lot of research and may pose problems with regards to patent and accessibility to the new plant species. However the solution of the nutrient deficiency issue may partially lie in the past. $S^0$ used to be more widely utilised as fungicide and fertilisers contained more impurities, such as S and Zn, while today, purer N fertilisers are preferred by farmers. Plant nutrition, as well as human nutrition, is a matter of the interaction between the numerous essential elements for life.

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# Appendix

## A Wheat growth stages

| Cereal grain development by Zadoks, Feekes and Haun | | | |
|---|---|---|---|
| **Zadoks** Scale | **Feekes** Scale | **Haun** Scale | Description |
| | | | **Germination** |
| 0 | - | - | Dry Seed |
| 1 | - | - | Start of imbibition |
| 3 | - | - | Imbibition complete |
| 5 | - | - | Radicle emerged from seed |
| 7 | - | - | Coleoptile emerged from seed |
| 9 | - | 0 | Leaf just at coleoptile tip |
| | | | **Seedling Growth** |
| 10 | 1 | - | First leaf through coleoptile |
| 11 | - | 1 | First leaf extended |
| 12 | - | 1.+ | Second leaf extending |
| 13 | - | 2.+ | Third leaf extending |
| 14 | - | 3.+ | Fourth leaf extending |
| 15 | - | 4.+ | Fifth leaf extending |
| 16 | - | 5.+ | Sixth leaf extending |
| 17 | - | 6.+ | Seventh leaf extending |
| 18 | - | 7.+ | Eighth leaf extending |
| 19 | - | - | Nine or more leaves extended |
| | | | **Tillering** |
| 20 | - | - | Main shoot only |
| 21 | 2 | - | Main shoot and one tiller |
| 22 | - | - | Main shoot and two tillers |
| 23 | - | - | Main shoot and three tillers |
| 24 | - | - | Main shoot and four tillers |
| 25 | - | - | Main shoot and five tillers |
| 26 | 3 | - | Main shoot and six tillers |
| 27 | - | - | Main shoot and seven tillers |
| 28 | - | - | Main shoot and eight tillers |
| 29 | - | - | Main shoot and nine tillers |
| | | | **Stem Elongation** |
| 30 | 4-5 | - | Psuedo stem erection |
| 31 | 6 | - | First node detectable |
| 32 | 7 | - | Second node detectable |
| 33 | - | - | Third node detectable |
| 34 | - | - | Fourth node detectable |
| 35 | - | - | Fifth node detectable |
| 36 | - | - | Sixth node detectable |

| | | | |
|---|---|---|---|
| 37 | 8 | - | Flag leaf just visible |
| 39 | 9 | - | Flag leaf ligule/collar just visible |
| | | | **Booting** |
| 40 | - | - | --- |
| 41 | - | 08. Sep | Flag leaf sheath extending |
| 45 | 10 | 9.2 | Boot just swollen |
| 47 | - | - | Flag leaf sheath opening |
| 49 | - | 10.1 | First awns visible |
| | | | **Inflorescence Emergence** |
| 50 | 10.1 | 10.2 | First spikelet of inflorescence visible |
| 53 | 10.2 | - | 1/4 of inflorescence emerged |
| 55 | 10.3 | 10.5 | 1/2 of inflorescence emerged |
| 57 | 10.4 | 10.7 | 3/4 of inflorescence emerged |
| 59 | 10.5 | 11 | Emergence of inflorescence completed |
| | | | **Anthesis** |
| 60 | 10.51 | 11.4 | Beginning of anthesis |
| 65 | - | 11.5 | Anthesis 1/2 completed |
| 69 | - | 11.6 | Anthesis completed |
| | | | **Milk Development** |
| 70 | - | - | --- |
| 71 | 10.54 | 12.1 | Kernel watery-ripe |
| 73 | - | 13 | Early milk |
| 75 | 11.1 | - | Medium milk |
| 77 | - | - | Late milk |
| | | | **Dough Development** |
| 80 | - | - | --- |
| 83 | - | 14 | Early dough |
| 85 | 11.2 | - | Soft dough |
| 87 | - | 15 | Hard dough |
| | | | **Ripening** |
| 90 | - | - | --- |
| 91 | 11.3 | - | Kernel hard (difficult to divide by thumbnail) |
| 92 | 11.4 | 16 | Kernel hard (can no longer be dented by thumbnail) |
| 93 | - | - | Kernel loosening in daytime |
| 94 | - | - | Overripe, straw dead and collapsing |
| 95 | - | - | Seed dormant |
| 96 | - | - | Viable seed giving 50% germination |
| 97 | - | - | Seed not dormant |
| 98 | - | - | Secondary dormancy induced |
| 99 | - | - | Secondary dormancy lost |

Source: http://plantsci.missouri.edu/cropsys/growth.html, 6.5.2011

## B Formulas

### *B.1 Soil texture analysis*

The percentage of particles with a diameter d is calculated as follows:

$$g(\%) = G_P \cdot K = G_P \cdot \frac{100 \cdot V_T}{V_P \cdot G_T}$$

$V_T$ Content of the cylinder (ml)

$V_P$ Removed sample quantity (ml)

$G_T$ Weighted sample (g)

$G_P$ Removed particle quantity (g)

$G_P$ is calculated via evaporation of $V_P$, weighing und subtraction of the calgon portion.

### *B.2 Soil organic carbon*

*Reactions*

$$2\ K_2Cr_2O_7 + 3\ C_{org} + 8\ H_2SO_4 \rightarrow 3\ CO_2 + 2\ Cr_2(SO_4)_3 + 2\ K_2SO_4 + 8\ H_2O$$

After completion of this reaction, the chromate that was not consumed is reduced with $Fe^{2+}$ to $Cr^{3+}$. As soon as Cr(VI) is reduced, Fe(II) appears in the solution and induces the colour change

$$K_2Cr_2O_7 + 6\ FeSO_4 + 7\ H_2SO_4 \rightarrow 3\ Fe_2(SO_4)_3 + Cr_2(SO_4)_3 + K_2SO_4 + 7\ H_2O$$

*Calculation*

The following formula is used to determine the percentage of organic carbon in the substrate:

$$\%_{org.C} = \frac{\left(10 - \frac{10 \cdot y}{x}\right) \cdot 300}{Boden(mg)}$$

*y: consumption of Fe(II) in the sample (mL)*

*x: consumption of Fe(II) in the sample (mL)*

*Boden: soil quantity*

Since not all the organic carbon can be detected in this method, the obtained value is multiplied by the correction factor 1.12. Soil organic matter is calculated by multiplying the corrected $C_{org}$ percentage by the factor 1.724.

## C Treatments, replicates and pot numbers

| Treatment | Pot number | Replicate |
|---|---|---|
| Control | 1 | 1 |
| Control | 2 | 2 |
| Control | 3 | 3 |
| Control | 4 | 4 |
| Control | 5 | 5 |
| Control | 6 | 6* (unplanted) |
| Zn | 7 | 1 |
| Zn | 8 | 2 |
| Zn | 9 | 3 |
| Zn | 10 | 4 |
| Zn | 11 | 5 |
| Zn | 12 | 12* (unplanted) |
| S | 13 | 1 |
| S | 14 | 2 |
| S | 15 | 3 |
| S | 16 | 4 |
| S | 17 | 5 |
| S | 18 | 18* (unplanted) |
| $SO_4$ | 19 | 1 |
| $SO_4$ | 20 | 2 |
| $SO_4$ | 21 | 3 |
| $SO_4$ | 22 | 4 |
| $SO_4$ | 23 | 5 |
| $SO_4$ | 24 | 24* (unplanted) |
| Zn+S | 25 | 1 |
| Zn+S | 26 | 2 |
| Zn+S | 27 | 3 |
| Zn+S | 28 | 4 |
| Zn+S | 29 | 5 |
| Zn+S | 30 | 30* (unplanted) |
| $Zn+SO_4$ | 31 | 1 |
| $Zn+SO_4$ | 32 | 2 |
| $Zn+SO_4$ | 33 | 3 |
| $Zn+SO_4$ | 34 | 4 |
| $Zn+SO_4$ | 35 | 5 |
| $Zn+SO_4$ | 36 | 36* (unplanted) |

## D Results

### *D.0 Total element content (XRF analysis) of initial soil*

| Element | Total content (mg/kg) | Standard error |
|---|---|---|
| Mg | 0.372 | 0.012 |
| Al | 2.277 | 0.052 |
| Si | 25.253 | 0.577 |
| P | 0.023 | 0 |
| S | 95.5 | 13.996 |
| Cl | 24.233 | 8.414 |
| K | 1.323 | 0.037 |
| Ca | 1.163 | 0.044 |
| Mn | 0.016 | 0 |
| Fe | 0.895 | 0.09 |
| Cu | 6.567 | 0.696 |
| Zn | 15.967 | 0.984 |
| Mo | 2.467 | 0.841 |
| Cd | 0.767 | 0.426 |
| Pb | 8.733 | 0.536 |

***D.1 Dry biomass of wheat parts at harvest***

| | Pot No. | Total dry biomass | Dry grains biomass | Dry shoot biomass |
|---|---|---|---|---|
| | | g | g | g |
| Control | 1 | 0.890 | | 0.890 |
| Control | 2 | 1.010 | | 0.910 |
| Control | 3 | 1.285 | 0.235 | 0.750 |
| Control | 4 | 0.600 | | 0.600 |
| Control | 5 | 1.712 | 0.692 | 0.660 |
| Zn | 7 | 1.674 | 0.574 | 0.730 |
| Zn | 8 | 0.770 | | 0.770 |
| Zn | 9 | 1.401 | 0.361 | 0.740 |
| Zn | 10 | 1.518 | 0.428 | 0.720 |
| Zn | 11 | 0.850 | | 0.850 |
| S | 13 | 11.428 | 1.708 | 7.090 |
| S | 14 | 9.159 | 3.909 | 3.050 |
| S | 15 | 12.072 | 1.682 | 7.410 |
| S | 16 | 10.186 | 4.756 | 3.130 |
| S | 17 | 11.562 | 3.672 | 5.660 |
| $SO_4$ | 19 | 8.500 | 2.530 | 3.540 |
| $SO_4$ | 20 | 9.940 | | 7.710 |
| $SO_4$ | 21 | 9.175 | 2.805 | 3.830 |
| $SO_4$ | 22 | 11.460 | | 8.740 |
| $SO_4$ | 23 | 8.860 | | 7.160 |
| Zn+S | 25 | 12.046 | 5.366 | 3.660 |

| | Pot No. | Total dry biomass | Dry grains biomass | Dry shoot biomass |
|---|---|---|---|---|
| Zn+S | 26 | 9.002 | 4.767 | 2.445 |
| Zn+S | 27 | 12.909 | 3.569 | 6.540 |
| Zn+S | 28 | 11.439 | 4.569 | 3.520 |
| Zn+S | 29 | 10.450 | 5.320 | 3.090 |
| $Zn+SO_4$ | 31 | 11.007 | 1.997 | 6.550 |
| $Zn+SO_4$ | 32 | 11.321 | 1.151 | 7.360 |
| $Zn+SO_4$ | 33 | 10.580 | 3.950 | 3.850 |
| $Zn+SO_4$ | 34 | 10.052 | 2.002 | 6.050 |
| $Zn+SO_4$ | 35 | 11.095 | 4.525 | 3.530 |

***D.2 pH ($CaCl_2$) of planted soils at harvest***

| Treatment | Pot No | pH |
|---|---|---|
| Control | 1 | 7.53 |
| Control | 2 | 7.83 |
| Control | 3 | 7.68 |
| Control | 4 | 7.82 |
| Control | 5 | 7.62 |
| Zn | 7 | 7.78 |
| Zn | 8 | 7.7 |
| Zn | 9 | 7.79 |
| Zn | 10 | 7.76 |
| Zn | 11 | 7.78 |
| S | 13 | 7.78 |
| S | 14 | 7.83 |
| S | 15 | 7.67 |
| S | 16 | 7.78 |
| S | 17 | 7.9 |
| $SO_4$ | 19 | 7.83 |
| $SO_4$ | 20 | 7.84 |
| $SO_4$ | 21 | 7.91 |
| $SO_4$ | 22 | 7.99 |
| $SO_4$ | 23 | 8.06 |
| Zn+S | 25 | 7.97 |
| Zn+S | 26 | 7.89 |
| Zn+S | 27 | 7.95 |

| | | |
|---|---|---|
| Zn+S | 28 | 7.88 |
| Zn+S | 29 | 7.91 |
| Zn+ $SO_4$ | 31 | 8.02 |
| Zn+ $SO_4$ | 32 | 7.84 |
| Zn+ $SO_4$ | 33 | 8.08 |
| Zn+ $SO_4$ | 34 | 7.87 |
| Zn+ $SO_4$ | 35 | 7.85 |

***D.3 pH ($CaCl_2$) with time***

| **Days after experiment start** | **10** | **23** | **44** | **73** | **121** |
|---|---|---|---|---|---|
| Control | 6.93 | 6.98 | 7.13 | 7.05 | 7.56 |
| Zn | 6.89 | 6.88 | 7.04 | 6.99 | 7.21 |
| S | 6.87 | 6.9 | 6.9 | 6.93 | 7.3 |
| SO4 | 7 | 7.01 | 7.12 | 7.15 | 7.55 |
| Zn+S | 6.84 | 6.82 | 6.9 | 6.93 | 7.27 |
| Zn+SO4 | 6.84 | 6.92 | 7.04 | 7.05 | 7.6 |

***D.4 Available sulphate in soil with time***

| **Days after experiment start** | **10** | **23** | **44** | **73** | **121** |
|---|---|---|---|---|---|
| Control | 4.2 | 0.0 | 6.1 | | 0.0 |
| Zn | 4.3 | 6.1 | 3.8 | 3.1 | 2.1 |
| S | 20.7 | 57.1 | 54.5 | 83.8 | 96.0 |
| SO4 | 166.8 | 221.8 | 337.5 | 97.4 | 88.9 |
| Zn+S | 9.1 | 34.3 | 54.1 | 62.5 | 417.8 |
| Zn+SO4 | 179.9 | 76.3 | 1080.0 | 60.0 | 343.7 |

***D.5 DTPA-available Zn in soil with time***

| **Days after experiment start** | 10 | 23 | 44 | 73 | 121 |
|---|---|---|---|---|---|
| Control | 0.22 | 0.14 | 0.22 | 0.26 | 0.50 |
| Zn | 1.06 | 0.99 | 0.90 | 1.03 | 1.23 |
| S | 0.24 | 0.18 | 0.17 | 0.30 | 0.33 |
| SO4 | 0.18 | 0.10 | 0.04 | 0.16 | 0.29 |
| Zn+S | 1.00 | 1.08 | 1.03 | 1.04 | 1.03 |
| Zn+SO4 | 0.89 | 0.84 | 0.76 | 0.77 | 0.85 |

***D.6 Plant height at harvest***

| | | **Tiller 1** | **Tiller 2** | **Tiller 3** | **Tiller 4** | **Tiller 5** | **Tiller 6** | **Pot average** |
|---|---|---|---|---|---|---|---|---|
| Treatment | Pot No. | (cm) | | | | | | |
| Control | 1 | 22 | | | | | | **22** |
| Control | 2 | 35 | | | | | | **35** |
| Control | 3 | 49 | | | | | | **49** |
| Control | 4 | 13 | 18 | | | | | **16** |
| Control | 5 | 51 | | | | | | **51** |
| Zn | 7 | 52 | | | | | | **52** |
| Zn | 8 | 22 | | | | | | **22** |
| Zn | 9 | 49 | | | | | | **49** |
| Zn | 10 | 48 | | | | | | **48** |
| Zn | 11 | 20 | | | | | | **20** |
| S | 13 | 63 | 63 | 59 | | | | **62** |
| S | 14 | 56 | 58 | 53 | | | | **56** |
| S | 15 | 72 | 57 | 64 | 44 | | | **59** |
| S | 16 | 54 | 61 | 62 | | | | **59** |
| S | 17 | 60 | 63 | 66 | | | | **63** |
| $SO_4$ | 19 | 61 | 69 | 56 | | | | **62** |
| $SO_4$ | 20 | 64 | 51 | 56 | 51 | | | **55** |
| $SO_4$ | 21 | 56 | 63 | 69 | | | | **63** |
| $SO_4$ | 22 | 72 | 58 | 73 | | | | **67** |
| $SO_4$ | 23 | 68 | 62 | 55 | | | | **62** |
| Zn+S | 25 | 64 | 48 | 59 | 54 | | | **56** |
| Zn+S | 26 | 38 | 49 | 44 | 31 | 45 | | **41** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Zn+S | 27 | 51 | 43 | 67 | 44 | 42 | 52 | **50** |
| Zn+S | 28 | 38 | 65 | 62 | 63 | | | **57** |
| Zn+S | 29 | 33 | 57 | 50 | 53 | | | **48** |
| $Zn+SO_4$ | 31 | 67 | 59 | 53 | 54 | | | **58** |
| $Zn+SO_4$ | 32 | 62 | 39 | 61 | 56 | | | **55** |
| $Zn+SO_4$ | 33 | 67 | 55 | 62 | | | | **61** |
| $Zn+SO_4$ | 34 | 59 | 57 | 52 | 50 | | | **55** |
| $Zn+SO_4$ | 35 | 50 | 59 | 59 | 61 | | | **57** |

***D.7 Seed statistics***

| **Treatment** | **Pot No** | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Head 1 | Head 2 | Head 3 | Head 4 | Head 5 | Head 6 | Number of seeds | Number of heads | Seeds per head | early head or late head? |
| Control | 1 | 0 | | | | | | 0 | 0 | 0.00 | - |
| Control | 2 | 0 | | | | | | 0 | 1 | 0.00 | late |
| Control | 3 | 6 | | | | | | 6 | 1 | 6.00 | early |
| Control | 4 | 0 | | | | | | 0 | 0 | 0.00 | - |
| Control | 5 | 20 | | | | | | 20 | 1 | 20.00 | early |
| Zn | 7 | 23 | | | | | | 23 | 1 | 23.00 | early |
| Zn | 8 | 0 | | | | | | 0 | 0 | 0.00 | - |
| Zn | 9 | 14 | | | | | | 14 | 1 | 14.00 | early |
| Zn | 10 | 18 | | | | | | 18 | 1 | 18.00 | early |
| Zn | 11 | 0 | | | | | | 0 | 0 | 0.00 | - |
| S | 13 | 13 | 3 | 8 | | | | 24 | 3 | 8.00 | late |
| S | 14 | 16 | 45 | 36 | | | | 97 | 3 | 32.33 | early |
| S | 15 | 0 | 0 | 7 | 18 | | | 25 | 4 | 6.25 | late |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| S | 16 | 51 | 49 | 36 | | | | 136 | 3 | 45.33 | early |
| S | 17 | 33 | 39 | 3 | | | | 75 | 3 | 25.00 | late |
| $SO_4$ | 19 | 9 | 45 | 36 | | | | 90 | 3 | 30.00 | early |
| $SO_4$ | 20 | 0 | 0 | 0 | 0 | | | 0 | 4 | 0.00 | late |
| $SO_4$ | 21 | 30 | 8 | 50 | | | | 88 | 3 | 29.33 | early |
| $SO_4$ | 22 | 0 | 0 | 0 | | | | 0 | 3 | 0.00 | late |
| $SO_4$ | 23 | 0 | 0 | 0 | | | | 0 | 3 | 0.00 | late |
| Zn+S | 25 | 8 | 43 | 41 | 57 | | | 149 | 4 | 37.25 | early |
| Zn+S | 26 | 36 | 20 | 25 | 7 | 6 | | 94 | 5 | 18.80 | early |
| Zn+S | 27 | 0 | 5 | 3 | 4 | 18 | 25 | 55 | 6 | 9.17 | late |
| Zn+S | 28 | 33 | 50 | 41 | | | | 124 | 4 | 31.00 | early |
| Zn+S | 29 | 6 | 28 | 33 | 20 | | | 87 | 4 | 21.75 | early |
| Zn+$SO_4$ | 31 | 4 | 11 | 6 | 14 | | | 35 | 4 | 8.75 | late |
| Zn+$SO_4$ | 32 | 0 | 13 | 4 | 4 | | | 21 | 4 | 5.25 | late |
| Zn+$SO_4$ | 33 | 40 | 42 | 51 | | | | 133 | 3 | 44.33 | early |
| Zn+$SO_4$ | 34 | 19 | 14 | 8 | 11 | | | 52 | 3 | 17.33 | late |
| Zn+$SO_4$ | 35 | 27 | 44 | 19 | 51 | | | 141 | 4 | 35.25 | early |

***D.8 Zn contents***

| | Pot No | Zinc in grains | Zinc in shoots | Estimated Zinc in hulls | Total Zn per plant | % of 2 mg added Zn | Total Zn in grains | Total Zn in shoot |
|---|---|---|---|---|---|---|---|---|
| | | mg/kg | mg/kg | mg/kg | mg | | mg | mg |
| Control | 1 | 0.00 | 27.25 | - | 0.024 | | 0.000 | 0.024 |
| Control | 2 | 0.00 | 19.11 | 19.11 | 0.019 | | 0.000 | 0.017 |
| Control | 3 | 34.88 | 8.28 | 8.28 | 0.017 | | 0.008 | 0.006 |
| Control | 4 | 0.00 | 28.22 | - | 0.017 | | 0.000 | 0.017 |
| Control | 5 | 26.57 | 7.80 | 7.80 | 0.026 | | 0.018 | 0.005 |
| Zn | 7 | 51.83 | 35.26 | 35.26 | 0.069 | 3.43 | 0.030 | 0.026 |
| Zn | 8 | 0.00 | 98.57 | - | 0.076 | 3.79 | 0.000 | 0.076 |
| Zn | 9 | 64.89 | 42.81 | 42.81 | 0.068 | 3.40 | 0.023 | 0.032 |
| Zn | 10 | 56.79 | 30.55 | 30.55 | 0.058 | 2.88 | 0.024 | 0.022 |
| Zn | 11 | 0.00 | 71.75 | - | 0.061 | 3.05 | 0.000 | 0.061 |
| S | 13 | 22.55 | 3.18 | 3.18 | 0.069 | | 0.039 | 0.023 |
| S | 14 | 10.30 | 3.72 | 3.72 | 0.060 | | 0.040 | 0.011 |
| S | 15 | 18.38 | 7.06 | 7.06 | 0.104 | | 0.031 | 0.052 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| S | 16 | 8.02 | 3.54 | 3.54 | 0.057 | | 0.038 | 0.011 |
| S | 17 | 11.97 | 3.43 | 3.43 | 0.071 | | 0.044 | 0.019 |
| $SO_4$ | 19 | 11.52 | 4.63 | 3.99 | 0.055 | | 0.029 | 0.016 |
| $SO_4$ | 20 | 0.00 | 5.05 | 7.40 | 0.055 | | 0.000 | 0.039 |
| $SO_4$ | 21 | 12.12 | 4.38 | 4.70 | 0.063 | | 0.034 | 0.017 |
| $SO_4$ | 22 | 0.00 | 5.04 | 4.33 | 0.056 | | 0.000 | 0.044 |
| $SO_4$ | 23 | 0.00 | 5.61 | 6.12 | 0.051 | | 0.000 | 0.040 |
| Zn+S | 25 | 24.94 | 5.24 | 5.24 | 0.169 | 8.44 | 0.134 | 0.019 |
| Zn+S | 26 | 32.42 | 9.83 | 9.83 | 0.196 | 9.81 | 0.155 | 0.024 |
| Zn+S | 27 | 31.25 | 6.77 | 6.77 | 0.175 | 8.74 | 0.112 | 0.044 |
| Zn+S | 28 | 29.40 | 6.55 | 6.55 | 0.179 | 8.96 | 0.134 | 0.023 |
| Zn+S | 29 | 26.27 | 5.36 | 5.36 | 0.167 | 8.36 | 0.140 | 0.017 |
| $Zn+SO_4$ | 31 | 51.45 | 5.52 | 5.52 | 0.152 | 7.62 | 0.103 | 0.036 |
| $Zn+SO_4$ | 32 | 46.81 | 6.62 | 6.62 | 0.121 | 6.06 | 0.054 | 0.049 |
| $Zn+SO_4$ | 33 | 22.49 | 6.42 | 6.42 | 0.131 | 6.57 | 0.089 | 0.025 |
| $Zn+SO_4$ | 34 | 28.97 | 5.77 | 5.77 | 0.104 | 5.22 | 0.058 | 0.035 |
| $Zn+SO_4$ | 35 | 21.06 | 4.57 | 4.57 | 0.125 | 6.27 | 0.095 | 0.016 |

Zn content in wheat hulls: Bold and italics = measured value. Other values are estimated: concentration in shoots = concentration in hulls

***D.9 S contents***

| | Pot No. | S in grains | S in shoots | Estimated S in hulls | Total S per plant | % of 100 mg added S | Total S in grains | Total S in shoots |
|---|---|---|---|---|---|---|---|---|
| | | mg/kg | mg/kg | mg/kg | mg | | mg | mg |
| Control | 1 | 0 | 530 | | 0.472 | | 0.00 | 0.47 |
| Control | 2 | 0 | 307 | 206 | 0.300 | | 0.00 | 0.28 |
| Control | 3 | 784 | 340 | 170 | 0.490 | | 0.18 | 0.26 |
| Control | 4 | 0 | 614 | | 0.369 | | 0.00 | 0.37 |
| Control | 5 | 731 | 288 | 144 | 0.748 | | 0.51 | 0.19 |
| Zn | 7 | 708 | 328 | 164 | 0.707 | | 0.41 | 0.24 |
| Zn | 8 | 0 | 311 | | 0.240 | | 0.00 | 0.24 |
| Zn | 9 | 748 | 308 | 154 | 0.544 | | 0.27 | 0.23 |
| Zn | 10 | 712 | 260 | 130 | 0.541 | | 0.30 | 0.19 |
| Zn | 11 | 0 | 280 | | 0.238 | | 0.00 | 0.24 |
| S | 13 | 3028 | 1328 | 664 | 16.3 | 16.3 | 5.17 | 9.42 |
| S | 14 | 2474 | 2696 | 1348 | 20.9 | 20.9 | 9.67 | 8.22 |

| | Pot No. | S in grains | S in shoots | Estimated S in hulls | Total S per plant | % of 100 mg added S | Total S in grains | Total S in shoots |
|---|---|---|---|---|---|---|---|---|
| | | mg/kg | mg/kg | mg/kg | mg | | mg | mg |
| S | 15 | 2859 | 1241 | 620 | 15.8 | 15.8 | 4.81 | 9.19 |
| S | 16 | 2084 | 2076 | 1038 | 18.8 | 18.8 | 9.91 | 6.50 |
| S | 17 | 2438 | 1320 | 660 | 17.9 | 17.9 | 8.95 | 7.47 |
| $SO_4$ | 19 | 2975 | 3063 | *1541* | 22.1 | 22.1 | 7.53 | 10.84 |
| $SO_4$ | 20 | 0 | 2178 | ***1824*** | 20.9 | 20.9 | 0.00 | 16.79 |
| $SO_4$ | 21 | 2912 | 3003 | ***1317*** | 23.0 | 23.0 | 8.17 | 11.50 |
| $SO_4$ | 22 | 0 | 1814 | ***1220*** | 19.2 | 19.2 | 0.00 | 15.86 |
| $SO_4$ | 23 | 0 | 2471 | ***1660*** | 20.5 | 20.5 | 0.00 | 17.69 |
| Zn+S | 25 | 2127 | 1905 | 953 | 21.3 | 21.3 | 11.41 | 6.97 |
| Zn+S | 26 | 2480 | 2798 | 1399 | 21.2 | 21.2 | 11.82 | 6.84 |
| Zn+S | 27 | 2447 | 1096 | 548 | 17.4 | 17.4 | 8.73 | 7.16 |
| Zn+S | 28 | 2359 | 1944 | 972 | 20.9 | 20.9 | 10.78 | 6.84 |
| Zn+S | 29 | 2224 | 1912 | 956 | 19.7 | 19.7 | 11.83 | 5.91 |
| Zn+$SO_4$ | 31 | 3122 | 1847 | 924 | 20.6 | 20.6 | 6.23 | 12.10 |
| Zn+$SO_4$ | 32 | 2727 | 1655 | 828 | 17.6 | 17.6 | 3.14 | 12.18 |

| | Pot No. | S in grains | S in shoots | Estimated S in hulls | Total S per plant | % of 100 mg added S | Total S in grains | Total S in shoots |
|---|---|---|---|---|---|---|---|---|
| | | mg/kg | mg/kg | mg/kg | mg | | mg | mg |
| $Zn+SO_4$ | 33 | 2183 | 2069 | 1035 | 19.5 | 19.5 | 8.62 | 7.97 |
| $Zn+SO_4$ | 34 | 2559 | 2005 | 1003 | 19.3 | 19.3 | 5.12 | 12.13 |
| $Zn+SO_4$ | 35 | 2010 | 1874 | 937 | 18.6 | 18.6 | 9.10 | 6.61 |

S content in wheat hulls: Bold and italics = measured value. Other values are estimated: concentration in hulls = 67% concentration in shoot if no grains and 50% of shoot if grains

***D.10 Other Elements in grains***

| Treatment | Pot No. | P | Mn | Mg | K | Fe | Cu | Ca |
|---|---|---|---|---|---|---|---|---|
| | | (mg/kg) | | | | | | |
| Control | 3 | ***5441*** | ***40.5*** | ***655.7*** | ***4520*** | ***45.8*** | 2.72 | ***382.3*** |
| Control | 5 | ***4757*** | ***32.6*** | ***594.6*** | ***4681*** | ***32.9*** | 2.99 | ***341.6*** |
| Zn | 7 | ***5454*** | ***42.9*** | ***594.8*** | ***5281*** | ***40.7*** | 2.15 | ***476.3*** |
| Zn | 9 | ***6077*** | ***49.2*** | ***728.8*** | ***4818*** | ***44.6*** | 2.52 | ***474.6*** |
| Zn | 10 | ***6112*** | ***51.5*** | ***697.8*** | ***4885*** | ***39.2*** | 2.27 | ***471.6*** |
| S | 13 | ***7164*** | ***86.4*** | ***711.5*** | ***5662*** | ***63.0*** | 7.85 | ***620.4*** |
| S | 14 | ***4638*** | ***60.3*** | ***578.6*** | ***4190*** | ***40.6*** | 6.73 | ***518.7*** |
| S | 15 | ***6595*** | ***87.8*** | ***626.9*** | ***4990*** | ***62.9*** | 6.17 | ***614.3*** |
| S | 16 | ***3667*** | ***56.9*** | ***452.7*** | ***4173*** | ***38.7*** | 4.70 | ***472.9*** |
| S | 17 | ***5451*** | ***66.4*** | ***455.9*** | ***6516*** | ***61.0*** | 5.15 | ***616.9*** |
| $SO_4$ | 19 | ***5068*** | ***47.2*** | ***587.5*** | ***4387*** | ***43.4*** | 5.14 | ***521.9*** |
| $SO_4$ | 21 | ***5525*** | ***42.3*** | ***587.4*** | ***4530*** | ***52.0*** | 5.48 | ***425.6*** |
| Zn+S | 25 | ***3890*** | ***52.6*** | ***463.8*** | ***4314*** | ***45.6*** | 3.19 | ***478.8*** |

| Treatment | Pot No. | P | Mn | Mg | K | Fe | Cu | Ca |
|---|---|---|---|---|---|---|---|---|
| Zn+S | 26 | ***4175*** | ***53.0*** | ***486.2*** | ***4224*** | ***44.9*** | 4.13 | ***609.0*** |
| Zn+S | 27 | ***6032*** | ***64.0*** | ***491.8*** | ***4918*** | ***126.0*** | 3.61 | ***545.1*** |
| Zn+S | 28 | ***4508*** | ***57.1*** | ***499.8*** | ***4655*** | ***121.5*** | 3.82 | ***514.5*** |
| Zn+S | 29 | ***4313*** | ***54.0*** | ***506.0*** | ***4482*** | ***41.9*** | 3.73 | ***556.6*** |
| $Zn+SO_4$ | 31 | ***7368*** | ***49.7*** | ***689.3*** | ***4870*** | ***54.4*** | 4.20 | ***472.0*** |
| $Zn+SO_4$ | 32 | ***6202*** | ***48.5*** | ***687.7*** | ***5405*** | ***58.6*** | 4.51 | ***555.0*** |
| $Zn+SO_4$ | 33 | ***4095*** | ***27.9*** | ***464.8*** | ***4648*** | ***30.4*** | 2.59 | ***442.2*** |
| $Zn+SO_4$ | 34 | ***5698*** | ***30.2*** | ***468.4*** | ***7291*** | ***41.8*** | 2.68 | ***504.6*** |
| $Zn+SO_4$ | 35 | ***4099*** | 24.4 | ***478.6*** | ***4811*** | ***33.4*** | 2.63 | ***360.8*** |

Bold and italics: measured value was over the calibration range

***D.11 Other elements in shoots and hulls for $SO_4$***

| Treatment | plant part | Pot No. | P | Mn | Mg | K | Fe | Cu | Ca |
|---|---|---|---|---|---|---|---|---|---|
| | | | (mg/kg) | | | | | | |
| Control | shoot | 1 | ***4099*** | ***262.6*** | ***884*** | ***27000*** | ***127.4*** | 3.26 | ***5376*** |
| Control | shoot | 2 | ***3046*** | ***314.7*** | ***607*** | ***24698*** | 19.9 | 3.00 | ***5060*** |
| Control | shoot | 3 | 2174 | ***188.9*** | ***415*** | ***22639*** | 23.3 | 2.12 | ***5144*** |
| Control | shoot | 4 | ***3996*** | ***299.7*** | ***1121*** | ***26224*** | ***38.0*** | 3.20 | ***5470*** |
| Control | shoot | 5 | 1737 | ***157.8*** | ***472*** | ***28827*** | 12.0 | 2.34 | ***4498*** |
| Zn | shoot | 7 | 1849 | ***230.7*** | ***519*** | ***26200*** | 17.9 | 2.25 | ***4652*** |
| Zn | shoot | 8 | ***4323*** | ***313.7*** | ***823*** | ***25939*** | 16.7 | 2.50 | ***4817*** |
| Zn | shoot | 9 | 1847 | ***288.6*** | ***636*** | ***19912*** | ***27.9*** | 2.45 | ***4843*** |
| Zn | shoot | 10 | 1688 | ***257.9*** | ***578*** | ***21332*** | 11.4 | 2.00 | ***4707*** |
| Zn | shoot | 11 | ***4600*** | ***238.5*** | ***683*** | ***24350*** | 18.4 | 2.24 | ***4650*** |
| S | shoot | 13 | 384 | ***59.6*** | ***362*** | ***13233*** | ***32.8*** | 3.20 | ***3056*** |
| S | shoot | 14 | 604 | ***89.1*** | ***407*** | ***24788*** | ***58.9*** | 3.54 | ***3919*** |
| S | shoot | 15 | 362 | ***55.9*** | ***339*** | ***13954*** | ***27.5*** | 3.15 | ***2871*** |

| Treatment | plant part | Pot No. | P | Mn | Mg | K | Fe | Cu | Ca |
|---|---|---|---|---|---|---|---|---|---|
| S | shoot | 16 | 284 | ***82.1*** | ***419*** | ***23971*** | ***42.9*** | 3.04 | ***3840*** |
| S | shoot | 17 | 331 | ***61.2*** | ***385*** | ***16313*** | ***29.1*** | 4.15 | ***3060*** |
| $SO_4$ | shoot | 19 | 406 | ***32.4*** | ***396*** | ***17704*** | ***48.8*** | 4.23 | ***3436*** |
| $SO_4$ | shoot | 20 | 863 | ***29.8*** | ***335*** | ***11550*** | ***33.8*** | 3.98 | ***2090*** |
| $SO_4$ | shoot | 21 | 391 | 23.5 | ***377*** | ***17356*** | ***40.1*** | 3.79 | ***3250*** |
| $SO_4$ | shoot | 22 | 1188 | 23.6 | ***402*** | ***11582*** | ***37.5*** | 4.26 | ***2147*** |
| $SO_4$ | shoot | 23 | 947 | ***33.8*** | ***414*** | ***11955*** | ***35.6*** | 4.44 | ***2386*** |
| Zn+S | shoot | 25 | 246 | ***71.3*** | ***315*** | ***22542*** | ***54.8*** | 2.41 | ***3982*** |
| Zn+S | shoot | 26 | 565 | ***91.9*** | ***334*** | ***26498*** | ***102.0*** | 2.92 | ***4482*** |
| Zn+S | shoot | 27 | 276 | ***57.4*** | ***305*** | ***12949*** | 19.7 | 1.66 | ***3200*** |
| Zn+S | shoot | 28 | 276 | ***76.2*** | ***288*** | ***24915*** | ***27.4*** | 2.15 | ***3908*** |
| Zn+S | shoot | 29 | 256 | ***72.3*** | ***310*** | ***23918*** | 18.3 | 2.02 | ***4105*** |
| Zn+$SO_4$ | shoot | 31 | 417 | 21.5 | ***302*** | ***11948*** | 20.1 | 2.26 | ***2397*** |
| Zn+$SO_4$ | shoot | 32 | 512 | 23.3 | ***273*** | ***10207*** | 23.3 | 2.07 | ***2201*** |
| Zn+$SO_4$ | shoot | 33 | 444 | 22.8 | ***326*** | ***16691*** | 22.7 | 2.54 | ***2963*** |
| Zn+$SO_4$ | shoot | 34 | 462 | 27.1 | ***350*** | ***12798*** | 18.4 | 2.28 | ***2440*** |

| Treatment | plant part | Pot No. | P | Mn | Mg | K | Fe | Cu | Ca |
|---|---|---|---|---|---|---|---|---|---|
| $Zn+SO_4$ | shoot | 35 | 213 | 16.4 | ***273*** | ***19548*** | 15.1 | 2.06 | ***2898*** |
| $SO_4$ | hulls | 19 | 213 | 16.4 | ***273*** | ***19548*** | 15.1 | 2.06 | ***2898*** |
| $SO_4$ | hulls | 20 | 993 | ***33.9*** | ***287*** | ***5436*** | ***78.8*** | 3.57 | ***1521*** |
| $SO_4$ | hulls | 21 | 2532 | ***56.3*** | ***435*** | ***5531*** | ***58.0*** | 3.61 | ***1477*** |
| $SO_4$ | hulls | 22 | 1095 | 21.3 | ***415*** | ***6500*** | ***69.2*** | 3.36 | ***1421*** |
| $SO_4$ | hulls | 23 | 2430 | ***38.6*** | ***337*** | ***4743*** | ***55.3*** | 2.71 | ***1286*** |

Bold and italics: measured value was over the calibration range

***D.12 Concentrations in planted soils***

| Treatment | Pot No | Zn | Fe | Cu | Mn | $SO_4$ |
|---|---|---|---|---|---|---|
| | | mg /kg dry soil (DTPA-TEA) | | | | mg/kg dry soil ($KH_2PO_4$) |
| Control | 1 | 0.39 | ***4.95*** | 0.40 | 1.45 | 0.00 |
| Control | 2 | 0.53 | ***5.46*** | 0.47 | 1.52 | 0.00 |
| Control | 3 | 0.32 | ***5.77*** | 0.48 | 1.51 | 2.16 |
| Control | 4 | 0.29 | ***5.70*** | 0.37 | 1.38 | 0.00 |
| Control | 5 | 0.36 | ***5.51*** | 0.38 | 1.40 | 1.96 |
| Zn | 7 | 1.03 | ***5.78*** | 0.43 | 1.49 | 0.00 |
| Zn | 8 | 1.04 | ***6.12*** | 0.37 | 1.67 | 0.00 |
| Zn | 9 | 0.94 | ***5.28*** | 0.40 | 1.50 | 2.02 |
| Zn | 10 | 1.00 | ***5.55*** | 0.40 | 1.50 | 2.12 |
| Zn | 11 | 1.02 | ***6.33*** | 0.45 | 1.67 | 2.80 |
| S | 13 | 0.32 | ***5.59*** | 0.39 | 2.04 | 107.49 |
| S | 14 | 0.25 | ***5.56*** | 0.32 | 1.90 | 116.62 |
| S | 15 | 0.24 | ***5.75*** | 0.41 | 2.18 | 18.68 |

| | | | | | | |
|---|---|---|---|---|---|---|
| S | 16 | 0.23 | ***5.91*** | 0.35 | 1.73 | 23.92 |
| S | 17 | 0.25 | ***5.58*** | 0.36 | 2.49 | 158.01 |
| $SO_4$ | 19 | 0.26 | ***5.35*** | 0.31 | 1.55 | 60.15 |
| $SO_4$ | 20 | 0.29 | ***5.31*** | 0.41 | 1.48 | 8.80 |
| $SO_4$ | 21 | 0.28 | ***5.90*** | 0.34 | 1.34 | 11.77 |
| $SO_4$ | 22 | 0.43 | ***5.37*** | 0.44 | 1.59 | 17.10 |
| $SO_4$ | 23 | 0.32 | ***5.77*** | 0.40 | 1.51 | 38.28 |
| Zn+S | 25 | 0.99 | ***5.68*** | 0.35 | 1.82 | 72.68 |
| Zn+S | 26 | 1.08 | ***5.97*** | 0.34 | 2.55 | 112.52 |
| Zn+S | 27 | 0.94 | ***6.10*** | 0.50 | 2.08 | 10.00 |
| Zn+S | 28 | 4.13 | ***5.21*** | 0.92 | 1.50 | 59.06 |
| Zn+S | 29 | 0.98 | ***5.57*** | 0.35 | 1.88 | 108.84 |
| Zn+ $SO_4$ | 31 | 1.02 | ***5.57*** | 0.40 | 1.57 | 42.51 |
| Zn+ $SO_4$ | 32 | 0.85 | ***6.01*** | 0.53 | 1.56 | 16.33 |
| Zn+ $SO_4$ | 33 | 0.80 | ***5.49*** | 0.31 | 1.33 | 37.67 |
| Zn+ $SO_4$ | 34 | 0.77 | ***5.36*** | 0.34 | 1.58 | 41.46 |
| Zn+ $SO_4$ | 35 | 1.02 | ***5.66*** | 0.36 | 1.46 | 17.70 |

Bold and italics: measured value was over the calibration range