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**Zinc limitation and toxicity in crops and
effects of Silicon in ameliorating stress response**

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Acknowledgements

Abstract

Agriculture is the primary food source for human sustainability. The improvement of food provision through agriculture represents a major topic for plant biology. In addition to drought, salinity and starvation, the remediation from soil contaminations by metalloids and heavy metals is important to guarantee food safety.

Metalloids are beneficial and necessary elements for higher plants at low concentrations, but high levels of these result severely toxic both for plants and humans. Soil contamination induced by an excess of some metalloids is a widespread problem over the world, causing economic disease and health threatens by human consumption.

In addition to metalloids, heavy metal pollution is rapidly increasing and present many environmental problems. Among heavy metals, some metals such as cadmium (Cd), lead (Pb) and chromium (Cr) have no known biological role while others such as copper (Cu), manganese (Mn) and zinc (Zn) are required in small amount for normal plant growth and developments but are extremely toxic to plants and animals slightly larger than the required concentrations.

While some metalloids and heavy metals effects are largely identified, a number of potential ameliorative effects of silicon (Si) are actually discussed. In fact, the silicon utilization as fertilizer is a recent suggestion to guarantee a compatible and sustainable agriculture inducing plant growth and to improve biotic and abiotic stress tolerance, such as metalloids deficiency or toxicity.

In the present doctoral thesis, we analyzed three horticultural plants of great agronomic importance, exhibiting different tolerance to Zn, *Lactuca sativa* cv. Phillipus, *Brassica oleracea* cv. Bronco, and barley (*Hordeum vulgare*). These species were exposed to Zn toxicity and deficiency in order to evaluate the contribution of compatible osmolytes, in the mechanism(s) of tolerance to Zn stress. As further control, the effects of a heavy metal such as cadmium were tested on barley plants in order to discriminate and confront the damages induced by polluting metals with those caused by limitation or excess of a nutritional microelement as zinc.

Furthermore, we studied the possible beneficial effect of Si on ameliorating plant stress conditions in *Hordeum vulgare*.

In conclusion, this project suggests that metals excess and/or deficiency induces in crops substantially similar responses depending on their capability to manage the stress induced by pollutants. Furthermore, stress symptoms induced by metals are clearly mitigated by Si supply, thus improving plant tolerance mechanisms such as photosynthesis and photorespiratory systems.

General introduction

Chapter 1 is proposed to cover a broad overview of traits and mechanisms known to play a role in metalloids excess tolerance in plants. Stress-induced regulatory genes, specifically involved in survival responses, in metabolism and development, are covered. Metalloids are beneficial as well as necessary elements for higher plants at low concentration but elevated concentration of some of these are greatly toxic both for plants and human. The soil contamination induced by an excess of arsenic (As), boron (Br), antimony (Sb) and other metalloids is a widespread problem over the world including USA, Europe and Asia. These conditions cause economic disease and health threatens by human consumption. Therefore, improving the knowledge on plant metalloids response would represent an essential topic for global food security. While some dangerous metalloids effects are largely identified, a number of potential ameliorative effects of silicon (Si) are actually discussed. In fact, the silicon utilization as fertilizer is a recent suggestion to guarantee a compatible and sustainable agriculture inducing plant growth and to improve biotic and abiotic stress tolerance. Further, a briefly dissection of beneficial molecular modification induced by Si is proposed.

Chapter 2 contains many aspect helpful to generate a better comprehension of silicon role in promoting sustainable development. Silicon is the second most abundant element in the Earth ecosystem, despite that silicon is not usually considered as an essential mineral nutrient for higher plants. However its beneficial role in alleviating biotic and abiotic stress has been well documented and Si has been used as an important fertilizer component.

In the last decades, several studies have been performed to explain how and why silicon presence in higher plants can enhance resistance and/or tolerance to abiotic stresses. Silicon alleviates stress stimulating antioxidant systems, carrying out a complexation or co-precipitation with toxic metal

ions, immobilizing toxic metal ions in growth media, interfering uptake processes, and with the compartmentation of metal ions within plants.

In **Chapter 3**, two horticultural plants exhibiting different tolerance to Zn, *Lactuca sativa* cv. Phillipus, and *Brassica oleracea* cv. Bronco, were exposed to Zn in order to evaluate the contribution of compatible osmolytes, in the mechanism(s) of tolerance to Zn stress.

The higher susceptibility of *L. sativa* to Zn stress is confirmed: lettuce plants experienced a strong reduction in biomass; in the meanwhile, the levels of proline (Pro) and glycine betaine (GB) increased. These results would suggest that in *L. sativa* the increase of Pro and GB does not represent a mechanism of resistance to toxicity, but it is likely a symptom of Zn-stress.

Conversely, in *B. oleracea*, a slight decrease in Pro levels, mainly catalysed by degradation through proline dehydrogenase (PDH), was observed; a similar behaviour affected GB levels. On the other hand, γ -aminobutyric acid (GABA) synthesis was slightly, but significantly, increased. The presence of high levels of GABA in Zn-stressed *B. oleracea* would suggest that reactive oxygen species (ROS) detoxification could be essential to improve the resistance to toxicity under metal stress conditions.

In **Chapter 4** the effect of different Zn nutrition regimes on barley plants were investigated by a number of physiological and biochemical parameters. Furthermore, the effects of Si supply were investigated on the same plants. Generally, Si induced a mitigation of stress effects induced by both Zn limitation and excess: a reduction of photorespiratory pathway and an improvement of photosynthetic efficiency were observed. Zn diverts the basal metabolism towards stress metabolism. In contrast, Si alleviated these stress effects by increasing the photorespiratory ammonium turnover and improving the cell redox balance: GSH/GSSG ratio became more favourable (reduced) and a general stabilization of the oxidative stress responding pathway was observed.

In **Chapter 5** the effects of a heavy metal such as Cadmium were tested on barley plants in order to discriminate and confront the damages induced by polluting metals with those caused by limitation or excess of a nutritional microelement as Zinc.

Cadmium represents one of the most toxic pollutants in plant ecosystems, causing plant growth inhibition, decrease in photosynthesis and changes in plant basal metabolism.

Barley plants exposed to Cd showed changes in pigments content, RubisCO large subunit (LS), and D1 protein, indicating a severe reduction in photosynthetic efficiency. Furthermore, the decrease of nitrate reductase activity and changes in free amino acids levels show a general stress condition of nitrogen assimilation. Cadmium increased the activities of ROS-scavenging enzymes; among these, ascorbate peroxidase rate was the most noticeably increased. It is worth noting that glucose-6-phosphate dehydrogenase, showed changes in both activities and occurrence during cadmium stress. These data suggest that G6PDH would modulate redox homeostasis under metal exposure, and possibly satisfy the increased request of reductants to counteract the oxidative burst induced by cadmium.

Therefore, the results suggest that APX and G6PDH may play a pivotal role to counteract the oxidative stress induced by cadmium in barley plants.

The **general conclusions** of this project suggest that the responses to metals excess and/or deficiency in crops look substantially similar and may be controlled in different species depending on their capability to manage the oxidative stress induced by pollutants.

Interestingly, Si mitigates stress symptoms induced by metals, improving photosynthesis and ameliorating photorespiratory response.

Chapter 1

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Molecular investigation on metalloid stress response in higher plants: recent insight from toxicity to tolerance

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Chapter 1

Molecular investigation on metalloid stress response in higher plants: recent insight from toxicity to tolerance

ABSTRACT

Metalloids are beneficial as well as necessary elements for higher plants at low levels, but high concentrations of these can be toxic both for plants and humans.

Soil contamination induced by an excess of arsenic (As), boron (B), antimony (Sb) and other metalloids is a widespread problem over the world including Europe, North America and Asia.

These conditions produce health threats by human consumption. Therefore, improving our knowledge about metalloid response in plants would represent an essential topic for global food security. While some dangerous effects of metalloids have been largely identified, a number of potential ameliorative effects of silicon (Si) are actually discussed. In fact, the use of Si as fertilizer can not only guarantee a sustainable agriculture, but also improve biotic and abiotic stress tolerance.

This chapter aims at presenting and discussing those traits and mechanisms known to play a role in metalloid excess tolerance in plants. Stress-induced regulatory genes, specifically involved in survival responses, in metabolism and development, will be covered. Further, a brief discussion of the beneficial effects promoted by Si is proposed.

INTRODUCTION

Agriculture is the primary food source for human sustainability (Tester and Langridge, 2010). Considering the increasing world population, the improvement of food provision through agriculture represents a major topic for plant biology researchers (Eckardt et al., 2009; Ruggiero et al., 2017). Consistently with drought, salinity and starvation, the remediation from soil contaminations by heavy metals and metalloids is an impelling challenge to guarantee food safety (Clements and Ma, 2016; Landi et al., 2017a). Particularly, metalloids are beneficial and essential elements for higher plants, but the contamination by these elements is a widespread problem in several countries in Europe, North America and Asia (Awashti et al., 2017). As a consequence, elements such as arsenic (As), boron (B), antimony (Sb) are commonly listed among the first 200 pollutants by the US Agency for Toxic Substances and Disease Registry in the Priority List of Hazardous Substances (ATSDR, 2017, <http://www.atsdr.cdc.gov/spl/index.html>). Similarly, the European Union and the World Health Organization (WHO) consider both As and Sb dangerous soil and food contaminants, being assessed that these may induce cancer and respiratory diseases (Ortega et al., 2017).

This chapter gives a broad overview of the genetic response in crops and model plants with the aim of inspiring future biotechnological strategies aimed at enhancing the response to metalloid excesses. A number of genes and key processes have been investigated to address the molecular basis of the metalloid tolerance. Furthermore, this chapter also addresses the beneficial effects induced by Si in the tolerance to metalloid toxicities.

METALLOIDS IN SOIL: UPTAKE AND DANGEROUS EFFECTS ON PLANTS

In recent years, metalloid and heavy metal contaminations have been characterized in many agricultural areas over the world, especially near industrial areas (Salem et al., 2017). This represents a critical problem for agriculture, food production and human health (Clements and Ma, 2016; Ortega et al., 2017). Toxic metalloids such arsenic (As) or antimony (Sb) are naturally present in the soil (Awasthi et al., 2017). More specifically, Sb is poorly present in the Earth's crust, ranging from 0.2 and 0.3 mg kg⁻¹; in contrast, Sb in soil is concentrated from 0.3 to 8.6 mg kg⁻¹ (Johnson et al., 2005). These levels could be increased by anthropogenic action; Sb levels greater than 1800 mg kg⁻¹ have indeed been measured around mine soils (Ortega et al., 2017). Similarly, WHO published guidelines with the permissible limit of As in drinking water (10mg/L), but in many developing countries, including Bangladesh and others of South Asia, the As levels in drinking water are 50mg/L (WHO guidelines, 2004). This results in serious consequences on the cultivation of rice (*Oryza sativa*), the crop most severely affected by As contamination, as compared to other cereal crops as wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and maize (*Zea mays*) (Awasthi et al., 2017). This is caused by the flooded cultivation of rice (Bakhat et al., 2017). As example, a soil As concentration >60 mg kg⁻¹ is sufficient to induce symptoms of poisoning by metalloids, such as stunted growth, brown spots and scorching on leaves (Khan et al., 2010); similar dangerous effects were also displayed for cultivation upon B excess exposure (Reid et al., 2004).

Both As and Sb exist in the environment in different inorganic and organic forms. Among the inorganic forms, we will hereafter consider arsenate As(V) and arsenite As(III), that are the most prevalent, as well toxic, inorganic forms for As (Awasthi et al., 2017); while Sb is present as antimonate Sb(V) and antimonite Sb(III) (Filella et al., 2007). These latter forms are adsorbed by plants from the environment. Other forms of As are monomethylarsonic acid MMA(V), dimethylarsinic acid DMA(V). As and Sb show similar chemical properties; they are easily soluble

as $\text{As}(\text{OH})_3$ and $\text{Sb}(\text{OH})_3$ and their pK_a values are 9.2 and 11.8 for As(III) and Sb(III), respectively. Based on these evidences, As and Sb are adsorbed by plants using similar absorption mechanism (Kamiya and Fujiwara, 2009). It should be noted that the chemical structures of As and Sb are quite similar with potassium, therefore these elements can be assimilated by plants using potassium channels, which are not able to discriminate the differences (Awashti et al., 2017).

Boron (B) is an essential micronutrient for plant development, growth and physiology (Yoshinari et al., 2016; Macho-Rivero et al., 2017; Tassi et al., 2017). This element is naturally present in soils as boric acid forms ($\text{pK}_a = 9.24$), and as borate. More specifically, borate plays an essential role in the cell wall structure, by forming cross-links with pectic polysaccharides (Wakuta et al., 2016). B uptake mechanism from soil to the plants remains controversial, but recent evidences suggested that this transport into plant cells could occur by direct permeation of the lipid bilayer, rather than using transport proteins (Fitzpatrick and Reid, 2009). B requirement for crops is variable among different species, but generally the optimum B content ranged from 20 to 100 mg Kg^{-1} (Tassi et al., 2017). Although B supply is essential for plants, an excess of B results in toxicity and other symptoms severely affecting crop production (Da Silva Leonardo et al., 2016).

The excess of As, Sb and B assimilation in plants induces various detrimental effects and damages in different cellular structures, tissues and organs. Plant exposed to toxic levels of heavy metals and metalloids showed similar primary responses, consisting in a quick production of reactive oxygen species (ROS), including superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl anion (OH^-) and singlet oxygen ($^1\text{O}_2$), as well as the formation of reactive nitrogen species (RNS), such as nitric oxide (NO), peroxynitrite (ONOO^-), S-nitrosothiols (RSNO_s) and S-nitrosoglutathione (GSNO) (Del Rio, 2015; Awashti et al., 2017; Landi et al., 2017a; Ortega et al., 2017; Soury et al., 2017). Moreover, it is worth to point out that increases in ascorbate peroxidase (APX), superoxide dismutase (SOD), peroxidase (POX), glutathione reductase (GR) and catalase (CAT) activities were reported in various crops and model plants upon both Sb and As exposure (Bakhat et al., 2017;

Bianucci et al., 2017; Chandrakar et al., 2017; Ortega et al., 2017; and others). Intriguingly, recent evidences showed, during metalloids exposition, an overall increase in the main component of the NADPH-generating system composed by glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase (6PGDH), NADP-malic enzyme (NADP-ME), and NADP-isocitrate dehydrogenase (ICDH) (Corpas et al., 2016). Similar responses were reported upon a wide range of abiotic stresses, such as salinity, drought and heat both for scavenging and NADPH-generating enzymes (Gill and Tuteja, 2010; Cardi et al., 2015; Sofu et al., 2015; Landi et al., 2016; Landi et al., 2017b). On the other hand, studies on sunflower showed that, contrarily to other metalloids, B deficiency induces ROS generation and changes in antioxidant enzyme activities, resulting in oxidative damage (El-Shinthawy, 1999).

Furthermore, another major cause for toxicity of metalloids is the interaction with sulfhydryl groups (Clemens and Ma, 2016). This interaction disturbs the proteins sulfhydryl bounds, thus inducing damages in protein structures and catalytic activities (Zhao et al., 2010). It has been demonstrated that high concentrations of As cause a critical depletion of glutathione S-transferase activity caused by the high affinity between sulfhydryl groups and As. This increases the oxidative stress caused by As, thereby provoking a rapid degradation of lipids, proteins and nucleic acids (Hernandez et al., 2015; Bianucci et al., 2017). Finally, it is worth to point out that metalloids interact with pathways involving lipid kinases (Escobar-Sepulveda et al., 2017). Particularly, As exposure induces the activation of phosphatidic acid pathway by increasing in Phospholipase D, Phospholipase C and Diacylglycerol kinase activities. This plays a critical role in the rapid stomata closure, reducing water loss by transpiration and inducing a minor absorption of As by roots (Armendariz et al., 2016).

Excess B provokes serious toxicity as well. This poisoning is caused by the interaction between borate with cis-diol containing substrates, such as ATP, NAD⁺, or RNA, thus restraining a number of cellular activities and inducing breaks in double-stranded DNA (Reid et al., 2004; Wakuta et al., 2016). Moreover, an excess of B in soil induces multiple roots disorder in many crops (e.g. tomato,

wheat, barley, grapevine and others): root growth inhibition, increase in suberin deposition, root morphology modification and less lateral root formation (Nable et al., 1997; Ghanati et al., 2002; Reid et al., 2004; Princi et al., 2016). Furthermore, recent evidences also demonstrate that B excess induces detrimental effects on nitrogen assimilation, by inhibiting NO_3 reduction, N organic concentration thus increasing GS/GOGAT and GDH activities (Cervilla et al., 2009; Princi et al., 2016).

STRESS RESPONSIVE GENES AGAINST METALLOIDS ACCUMULATION: AN OVERVIEW

In recent years, an increasing number of genes involved in response and tolerance to metalloid excess has been identified in crops and model plants. Some of these are listed in Table 1. The first process that scientists tried to manipulate was metalloid assimilation from soils. Usually, plants acquire beneficial and deleterious (e.g. As or Sb) elements using the same (or similar) transporters (Awasthi et al., 2016). Particularly, the chemical forms of As and Sb in aqueous solution make them transportable by aquaglyceroporins and hexose transporters. Further, As could be taken up by phosphate transporters (Dziubinska et al., 2012). It is worth to point out that As uptake is constrained by phosphate while plants with a constitutive reduction of expression of high affinity phosphate transporters showed an As-tolerant phenotype (Abedin et al., 2002).

Considering this, interesting results about As tolerance were recently obtained by the downregulation of phosphate transporters (PHTs) both in *A. thaliana* and rice (Wu et al., 2011; Kamiya et al., 2013; Awasthi et al., 2016). *AtPHT1;1*, *AtPHT1;4*, *AtPHT1;5*, *AtPHT1;6*, *AtPHT1;7*, *AtPHT1;8*, *AtPHT1;9* were recently identified to play a central role in phosphate uptake as well as As adsorption (Awasthi et al., 2016). Particularly, *AtPHT1;1* and *AtPHT1;7* were identified as important players in As sensitivity in *Arabidopsis thaliana* (Le Blanc et al., 2013). As a matter of fact, *A. thaliana* overexpressing these two phosphate transporters showed hypersensitivity to arsenate by an increased uptake of As. Intriguingly, the co-overexpression of *AtPHT1;1* and/or *AtPHT1;7* with *AtYCF1*, an ABC transporter (ATP-binding cassette transporter), suppressed the arsenate sensitivity by increasing vacuolar sequestration of the toxic element. Similar beneficial effects in As tolerance were also obtained in rice using *OsABC5* and *OsABC6* (Zhang et al., 2016).

Interesting results about phosphate transporters were also reported in rice for *OsPHT1;1* and *OsPHT1;8* (Wu et al., 2011; Kamiya et al., 2013). Naturally allelic variations in phosphate transporters were also discovered in different rice varieties, as Kasalath and Nipponbare. The

Kasalath genotype showed a naturally modified expression of two phosphate transporters *OsPT2* and *OsPT8* thus increased tolerance to As(V) contamination compared with Nipponbare (Wang et al., 2016).

Furthermore, the regulation of the localization and the expression of metalloids transporters are also important to induce an increased tolerance (Awasthi et al., 2016). For example *AtWRKY6* showed a role in arsenate assimilation by the regulation of *AtPHT1;1* transporter (Castrillo et al., 2013).

Analogous results were recently obtained for tolerance to the excess of B in *A. thaliana* and various crops (e.g. barley, wheat and tomato). Example of critical B transporters acting in B excess tolerance are *AtBOR1* (Yoshinari et al., 2016), *AtBOR4* (Miwa et al., 2007), *HvBOR4* (Reid, 2007), *SIBOR1*, *SIBOR4* (Princi et al., 2016), and *TaBOR4* (Reid, 2007). Plants can assimilate boron from soil by passive diffusion of boric acid across membranes and by boric acid channels. These assimilation processes are required for normal growth under low-B conditions (Wakuta et al., 2016). Passive diffusion of B depends by his theoretical lipid permeability coefficient ($8 \times 10^{-6} \text{ cm s}^{-1}$ - Raven, 1980). This particular value allows a constitutive passive diffusion through the lipid bilayer.

The physiological bases for tolerance to B excess are mediated by efflux transporters (Princi et al., 2016). In this context, a major role is played by *BOR1* and its paralogue *BOR4*. These proteins are responsible for directional B export from plants to the soil (Takano et al., 2002; Miwa et al., 2007; Princi et al., 2016). In fact, *A. thaliana* and rice overexpressing *AtBOR4* showed an increase tolerance to B excess (Kajikawa et al., 2011).

Moreover, B-tolerant tomato genotype (*Solanum lycopersicum*, cultivar Losna) showed a different pattern of expression of *SIBOR1* and *SIBOR4* than a B-susceptible genotype (*Solanum lycopersicum*, cultivar Ikram) under B stress (Princi et al., 2016). Recent evidences using GFP localized *AtBOR1* in the inner/stele-side domain of the plasma membrane in roots, cotyledons and hypocotyls. Intriguingly, the localization and the endocytosis of *AtBOR1* are regulated by the dynamin-related protein 1A (*AtDRP1* – Yoshinari et al., 2016). In addition, a channel-mediated

membrane B transport is required in root cells. Particularly, the *NIP5;1* gene encoding for the crucial channel involved in B deficiency uptake (Takano et al., 2006). This is a major intrinsic protein (MIP) showed a key role in B uptake (Takano et al., 2008). The distribution of B from roots to shoots mainly depends by plant species. In fact, B could arrange complex with sugar such as mannitol, sorbitol or fructose and this ability regulated the B translocation in different species of plant (Takano et al., 2008). *A.thaliana-BOR1* defective plants also showed a reduced translocation of B in xylem, inflorescences and young leaves (Takano et al., 2002).

The accumulation of metalloids from tissues into the grain of crops is another important process to be investigated (Awasthi et al., 2016). Recently, an *Oryza sativa* peptide transporter *OsPTR7* (also known as *OsNPF8.1*) has been identified as regulator of As transporter in rice grain (Tang et al., 2017). This gene showed a role in the transport of As from root to shoot and finally into grains; *OsPTR7*-knock out plants showed a reduction in grain As content (Tang et al., 2017).

Acquaporins play a role in metalloid transport, sequestration and tolerance (Fitzpatrick and Reid, 2009; Kamiya and Fujiwara, 2009; Awasthi et al., 2017). This family is divided into different sub-groups including tonoplast intrinsic proteins (TIPs), nodulin-26-like intrinsic proteins (NIPs), plasma membrane intrinsic proteins (PIPs) and others (Souri et al., 2017). An interesting candidate gene acting in metalloid tolerance is *AtNIP1;1*. This gene showed a central role both in Sb and As uptake (Kamiya and Fujiwara, 2009). T-DNA insertion in different NIPs of *A. thaliana*, including *AtNIP1;1*, *AtNIP1;2*, *AtNIP1,5* indicate that *AtNIP1;1* plays a major role in As and Sb response, increasing tolerance in both roots and shoots; similarly, the regulation of expression of *AtNIP1;1* is involved in tolerance to As (Awasthi et al., 2017).

The Calcium dependent kinase *AtCPK31* was recently identified as regulator of *AtNIP1;1* *A. thaliana* *AtCPK31*-Knock out plants showed a similar phenotype compared to *A. thaliana AtNIP1;1* knock-out plants. The double mutants, showed more effective responses in As tolerance compared with single mutants (Ji et al., 2017).

Table 1 – List of genes conferring metalloids stress tolerance.

Gene	Function	Metalloid	Regulation	Plant	References
<i>AtBOR1</i>	Borate efflux transporter	B	Up	<i>A.thaliana</i>	Takano et al., 2002
<i>AtBOR4</i>	Boron transporter	B	Up	<i>A.thaliana</i>	Miwa et al., 2007
<i>AtCPK31</i>	Calcium dependent protein kinase	As	Down	<i>A.thaliana</i>	Ji et al., 2017
<i>AtDRP1A</i>	Dyamin-related protein	B	Down	<i>A.thaliana</i>	Yoshinari et al., 2016
<i>AtIPK1</i>	Inositol pentakisphosphate 2-kinase	As	Up	<i>A.thaliana</i>	Sun et al., 2016
<i>AtNIP1;1</i>	Nodulin intrinsic protein	Sb	Down	<i>A.thaliana</i>	Kamiya and Fujiwara, 2009
<i>AtNIP5;1</i>	Boric acid canne	B	Up	<i>A.thaliana</i>	Takano et al., 2002
<i>AtNCED3</i>	9-cis epoxy-carotenoids dioxygenase	B	Up	<i>A.thaliana</i>	Macho-Rivero et al., 2017
<i>AtNUDX19</i>	Nudix hydrolase	As	Down	<i>A.thaliana</i>	Corpas et al., 2016
<i>AtPHT1;1</i>	Phosphate transporter	As	Down	<i>A.thaliana</i>	Awasthi et al., 2016
<i>AtPHT1;4-9</i>	Phosphate transporter	As	Down	<i>A.thaliana</i>	Awasthi et al., 2016
<i>AtWRKY6</i>	Transcription factor	As	Up	<i>A.thaliana</i>	Castrillo et al., 2013
<i>AtYCF1</i>	ABC transporter	As	Up	<i>A.thaliana</i>	Le Blanc et al., 2013
<i>HaGST</i>	Glutathione-S-transferase	Sb	Up	Sunflower	Ortega et al., 2017
<i>HaCuZnSOD</i>	Superoxide dismutase	Sb	Up	Sunflower	Ortega et al., 2017
<i>HvBOR2</i>	Boron transporter	B	Up	Barley	Reid, 2007
<i>HvBOR4</i>	Boron transporter	As	Up	Barley	Reid, 2007
<i>HvNIP1;2</i>	Nodulin intrinsic protein	As	Down	Barley	Katsuhara et al., 2014
<i>HvPIP1;3</i>	Plasma membrane intrinsic protein	As	Down	Barley	Fitzpatrick and Reid, 2009
<i>HvPIP1;4</i>	Plasma membrane intrinsic protein	As	Down	Barley	Fitzpatrick and Reid, 2009
<i>OsABCC1</i>	ABC-phytochelatin transporter	As	Up	Rice	Clemens and Ma, 2016
<i>OsABC5-6</i>	ABC transporter	As	Up	Rice	Zhang et al., 2016
<i>OsARM1</i>	R2R3 Myb Trascription factor	As	Down	Rice	F.Z. Wang et al., 2017
<i>OsGRX_G2.1</i>	Glutaredoxin	As	Up	Rice	Verma et al., 2016
<i>OsGRX_G7</i>	Glutaredoxin	As	Up	Rice	Verma et al., 2016
<i>OsHAC1;1-2</i>	Arsenate reductase	As	Up	Rice	Shi et al., 2016

<i>OsLsi1-2</i>	Silicon transporter	As	Down	Rice	F.Z. Wang et al., 2017
<i>OsLsi6</i>	Silicon transporter	As	Down	Rice	F.Z. Wang et al., 2017
<i>OsNIP3;2</i>	Nodulin intrinsic protein	As	Down	Rice	Chen et al., 2017
<i>OsNIP3;3</i>	Nodulin intrinsic protein	As	Down	Rice	Katsuhara et al., 2014
<i>OsPCS1</i>	Phytochelatin synthase	As	Up	Rice	Zhang et al., 2016
<i>OsPCS3</i>	Phytochelatin synthase	As	Up	Rice	Zhang et al., 2016
<i>OsPCS13</i>	Phytochelatin synthase	As	Up	Rice	Zhang et al., 2016
<i>OsPIP2;4</i>	Plasma membrane intrinsic protein	As	Down	Rice	Ma et al., 2012
<i>OsPIP2;6-7</i>	Plasma membrane intrinsic protein	As	Down	Rice	Ma et al., 2012
<i>OsPTR7</i>	Peptide transporter	As	Down	Rice	Tang et al., 2017
<i>OsPHT1;1</i>	Phosphate transporter	As	Down	Rice	Kamiya et al., 2013
<i>OsPHT1;8</i>	Phosphate transporter	As	Down	Rice	Wu et al., 2011
<i>OsTIP4;2</i>	Tonoplast intrinsic proteins	As	Down	Rice	Awasthi et al., 2016
<i>SIBOR1</i>	Boron transporter	B	Up	Tomato	Princi et al., 2016
<i>SIBOR4</i>	Boron transporter	B	Up	Tomato	Princi et al., 2016
<i>TaBOR4</i>	Boron transporter	B	Up	Wheat	Reid, 2007

Beneficial effects using NIPs manipulation were also reported for arsenite tolerance in rice and barley using *OsNIP2;3*, *OsNIP3;3* and *HvNIP1;2* (Katsuhara et al., 2014; Chen et al., 2017). Particularly, *OsNIP2;3* has recently been identified in rice primary and lateral roots and the mutated allele of this genes showed a reduced activity in As assimilation (Chen et al., 2017).

Intriguingly, a number of NIPs of rice (*OsNIP2.1* and *OsNIP2.2*) were also detected as As and silicon transporters and named *OsLSII* and *OsLSI6*, respectively (Awasthi et al., 2017; Wang et al., 2017). Recently, a transcription factor regulating the expression of *OsLSII* and *OsLSI6* was detected and identified as *OsARMI*, an R2R3 Myb transcription factor playing a central role in As stress response (Wang et al., 2017). Using a CRISP/Cas9 approach, different lines of rice plants were generated. These *OsARMI*-overexpressing plants showed an increased As sensitivity, while *OsARMI*-knock out plants showed opposite behaviors and an increased As stress tolerance. These evidences suggest that this transcription factors effectively plays essential functions in As uptake and in translocation from root to shoot (Wang et al., 2017).

Similar results on B susceptibility were obtained using *HvPIP1;3* and *HvPIP1;4* (Fitzpatrick and Reid, 2009). The expression of plasma membrane intrinsic proteins *HvPIP1;3* and *HvPIP1;4* in yeast complementation assays resulted in increased B sensitivity; differently, *OsPIP2;4*, *OsPIP2;6* and *OsPIP2;7* showed peculiar behavior favoring As tolerance (Mosa et al., 2012). Heterologous expression of these aquaporins in *Xenopus laevis* oocyte increased arsenite uptake; *OsPIP2;4*-*OsPIP2;6* and *OsPIP2;7*-overexpressing *A.thaliana* plants showed an enhanced tolerances to As and biomass accumulation thus reducing the As accumulation in roots and shoots. Considering these results, the authors suggested a bidirectional arsenite permeability of rice PIPs in plants (Mosa et al., 2012).

An important strategy involved in metalloid tolerance is sequestration in the vacuole. This process is mediated by a number of proteins and particularly by sulfur metabolism (Awasthi et al., 2017). This latest aspect plays a crucial role in As stress response, by the activation of –SH containing peptides as glutathione (GSH) or phytochelatins (PCs – Baptista et al., 2014). It has been recently

suggested that high sulfur supply induces an increased expression of PCs synthase genes (PCS), thus contributing to sequestration of As in the vacuole (Awasthi et al., 2017). In order to sustain metalloids sequestration PCs bind free As or Sb in the cytosol, thus creating complexes recognized by ABC transporters on the vacuolar membrane, finally increasing metalloids tolerance. Particularly, *OsPCS1*, *OsPCS3* and *OsPCS13* were recently identified as important actors in As tolerance in Rice (Zhang et al., 2016). Furthermore, an interesting up-regulation of the *Oryza sativa* ABC-phytochelatin complex transporter (*OsABCC1*) was recently reported in As tolerance (Clemens and Ma, 2016). Similarly to sulfur, phosphate homeostasis plays a role in metalloid tolerance as well (Sun et al., 2016; Awasthi et al., 2017). Recently, the inositol pentakisphosphate 2-kinase of *A. thaliana* (*AtIPK1*), was suggested to be a regulator between phosphate homeostasis and As detoxification in plants (Sun et al., 2016). *A. thaliana AtIPK1*-knock out plants showed a reduced tolerance against As exposure and an increased phosphorus starvation; as expected, the overexpression of this gene reverted the phenotype (Sun et al., 2016).

The increase of activities of scavenging enzymes represents a different approach to improve metalloid tolerance. Plants exposed to metalloids showed a rapid production of ROS (Awasthi et al., 2017; Souri et al., 2017). Sunflower (*Heliantus annuus*) plants exposed to Sb increased expression and activities of SOD (superoxide dismutase) and GST (Glutathione-s-transferase) (Ortega et al., 2017). Furthermore, NADPH and NADPH-generating enzymes were also recently identified as key factors in metalloid stress response to sustain the ROS scavenging system (Corpas et al., 2016; Ruiz-Torres et al., 2017). Interesting results in As tolerances were recently obtained using transgenic knock-out *A. thaliana* plants with a T-DNA insertion in the nudix hydrolases 19, *AtNUDX19* (Corpas et al., 2016). This enzyme belongs to a family characterized by the presence of a conserved sequence GX₅-EX₇-REUXEEXGU, possessing a NAD(P)H pyrophosphatase activity (Yoshimura and Shigeoka, 2015). The increase in G6PDH and 6PGDH activities in knock-out mutants both in control and As exposure resulted in an increased tolerance to metalloids. This evidences corroborate the idea of the main role played by the Oxidative pentose phosphate

pathway (OPPP) in plants subjected to various abiotic stresses (Cardi et al., 2011; Esposito et al., 2016; Landi et al., 2016).

Glutaredoxins include cysteine-rich and low molecular weight proteins involved in the maintenance and regulation of cellular redox state and in a number of various cellular processes (Lilling et al., 2008). *A.thaliana* transgenic plants overexpressing *OsGRX_C2.1* and *OsGRX_C7* showed an increased tolerance upon As exposure (Verma et al., 2017). Particularly, *OsGRXC2.1/7* overexpressing plants showed high glutathione levels, increased by As exposure, thus contrasting the detrimental effects of this metalloid. Overexpressing plants showed an interesting co-expression of *AtNIP1;1*, *AtNIP2;1* and *AtNIP7;1* suggesting that *OsGRXs* play a role in As tolerance both by the reduction of As(V) and creating a gradient of As(III) extruded through aquaporins, in their turn expressed at higher levels (Verma et al., 2017).

Phytohormones are involved in metalloid response. Recent evidences (Aquea et al., 2012) in *A. thaliana* highlighted the contribution of several genes involved in ABA synthesis, transduction and signaling in response to B toxicity. More recently, micro-array and RT-PCR analyses showed the up-regulation of *NCED3* (9-cis-epoxycarotenoid dioxygenase), *ABI1* and *AB2* genes (Protein phosphatases 2C) in *A. thaliana* exposed for 6h to B toxicity, thus increasing ABA synthesis and reducing transpiration rate (Macho-Rivero et al., 2016). The effects of B toxicity in wild-type *A. thaliana* and in *AtNCED3* knock-out plants resulted in higher transpiration rate, and stomatal conductance in transformed plants, thus accumulating a high B content in shoot with respect to wild-type plants.

A crucial role in As tolerance was reported for *Loc_Os02g01220* and *Loc_Os04g17660* encoding for two rhodanese-like proteins. These proteins showed an arsenate reductase activity and a 90% similarity with the *AtHAC1*, therefore these two genes have been recently renamed as *OsHAC1;1* and *OsHAC1;2*, respectively (Shi et al., 2016). A crucial aspect of As detoxification is the reduction from As(V) to As(III) because this can be extruded more easily than As(V) (Zhao et al., 2010).

Intriguingly, transgenic rice overexpressing *OsHAC1;1* and *OsHAC1;2* showed an increased As(III) efflux and a reduction in As accumulation, while knock-out plants were more susceptible upon As exposure and showed a decreased activity in the reduction of arsenate to arsenite (Shi et al., 2016).

METALLOIDS VS METALLOIDS: THE BENEFICIAL EFFECTS OF SILICON

Dangerous metalloid effects have been largely identified (Clemens and Ma, 2016; Awasthi et al., 2017) but metalloids also play beneficial effects on plant physiology. Silicon (Si), after oxygen, is the second most abundant elements in the world and a number of potential ameliorative effects of Si has been recently discussed (Landi et al., 2017; Luyckx et al., 2017). Si is not generally considered an essential element for plant growth, physiology and development, but recent evidences suggest that Si has beneficial effects and is able to improve biotic and abiotic stress tolerance thereby ensuring a compatible and sustainable agriculture (Guerriero et al., 2016; Landi et al., 2017). An emerging role for Si in plant response against metalloid toxic levels has been recently proposed (Deng et al., 2013; Pandey et al., 2016 Zhang et al., 2017).

Recently, Si fertilization showed promising results in arsenic tolerance in rice plants (Wu et al., 2015). The tolerance mechanisms to arsenic include the production of adventitious roots and induction of a barrier to radial oxygen loss (ROL – Wu et al., 2011) and ROL was defined as the ability to transfer oxygen from the aerenchyma to the rhizosphere (Deng et al., 2003). Screening a number of Chinese rice-genotypes with different ROL revealed that Si fertilization significantly reduced As accumulation both in shoots and roots of each genotype. Furthermore, rice genotypes with higher ROL were positively affected by Si, showing an enhanced ability to reduce As accumulation in the shoot. Previous investigations reported that Si decreased inorganic As accumulation in rice grain (Li et al., 2009) and this effect could be increased by combining Si fertilization with the selection of genotypes with high ROL (Wu et al., 2015); similar results were recently obtained in rice plants upon Sb exposure (Zhang et al., 2017). Upon Si nutrition, different rice genotypes showed increase in root, straw, husk and grain biomasses. The genotypes with lower

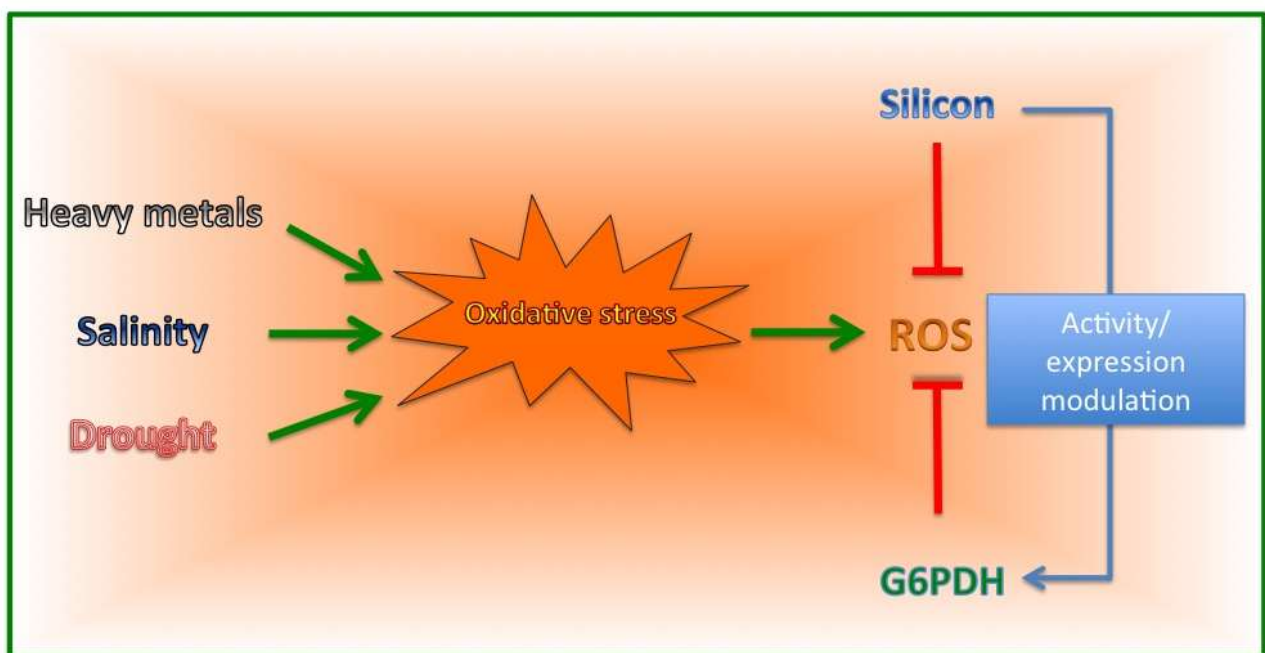
ROL production showed the highest accumulation of Sb, thus highlighting the correlation between ROL and Si nutrition in order to contrast and/or to alleviate metalloid toxicity.

Analogously, Si beneficial effects on rice grown upon arsenic exposure induced modification in the photosynthetic apparatus (Sanglard et al., 2014). Using two genotypes of rice, a wild type cultivar (*Oryza sativa* cv *Oochikara*) and its *OsLSII* mutant (defective in a Si transporter), a beneficial interaction between Si nutrition and the photosynthetic performance was observed. Arsenic exposure in rice plants induced a significant decrease of photosynthetic assimilation rate, mainly caused by a reduction in stomatal conductance and in CO₂ diffusion from stomata to mesophyll. Contrarily, Si supply induced ameliorative effects, by decreasing arsenic concentration in leaves and preserving the photosynthetic metabolism

(Sanglard et al., 2014). Particularly, the Si transporters *LSII* and *LSI2* play a major role in Si translocation from epidermis to steles in roots and then to the shoot (J.F. Ma et al., 2006). Recently, a second action of these transporters was observed in As accumulation, suggesting that As and Si shares similar assimilation pathways (J.F. Ma et al., 2008). Based on these evidences, the increasing of Si concentration induced a decrease of As accumulation in plants by a competitive inhibition (Zhao et al., 2010).

Moreover, metalloid toxicity induces an increased ROS production and show increased activity of scavenging enzymes (Del Rio, 2015; Awashti et al., 2017; Bakhat et al., 2017; Bianucci et al., 2017; Ortega et al., 2017). More recently, the involvement of Si in favouring a more efficient antioxidant response has been demonstrated (Gong et al., 2005; Guerriero et al., 2016; D. Ma et al., 2016; Pandey et al., 2016; Landi et al., 2017a). *Brassica juncea* exposed to As showed detrimental effects, inhibiting primary root growth, reducing the number of lateral roots, decreasing the assimilation of essential nutrients and activating the scavenging antioxidant system. Furthermore, an increase of H₂O₂ and malondialdehyde (MDA) was reported, highlighting oxidative stress and lipid peroxidation (Pandey et al., 2016). Intriguingly, As exposed plants upon Si nutrition showed similar behaviors to the control plants, limiting H₂O₂ and MDA increases; CAT, APX and SOD activities

were reduced, thus suggesting the involvement of Si in the regulation of the scavenging system upon oxidative stress (Pandey et al., 2016). Similar results were also shown in maize subjected to Sb stress and exposed to Si (Vaculikova et al., 2014): CAT, APX and GPOX (Guaiacol peroxidase) activities were increased upon Sb exposure while upon Si nutrition the activities of CAT, APX and GPOX returned at control levels. This behavior confirms the role of Si in the oxidative stress response (Vaculikova et al., 2014).



CONCLUSIONS

As discussed in this chapter, a number of key genes control or contribute to the response to toxic concentrations of metalloids. Understanding the molecular and physiological mechanism regulated by these genetic traits is essential for the genetic improvement of crops. Although model plants as *A. thaliana* remain the best system to improve our knowledge about metalloid response, new insights using crop models, especially rice (*Oryza sativa*), represent the best way for identifying new traits and finding processes for effective field application. Actually, new genomes and transcriptomes emerge daily for an increasing number of new species, but a thorough functional characterization is necessary in order to take advantage of the benefits this knowledge offers. Finally, Si nutrition was already extensively shown to be important in the protection against various abiotic stresses, and the use of this elements against toxic concentrations of metalloids could be provide additional sustainable agricultural practices.

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Chapter 2

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Roles of Silicon in alleviating Zinc stress in plants

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Chapter 2

Roles of Silicon in alleviating Zinc stress in plants

ABSTRACT

Silicon (Si) represents the second most diffused element on Earth; despite its abundance, it is generally considered non essential for most higher plants. However, its beneficial role in alleviating biotic and abiotic stress has been well documented and Si has been used as an important fertilizer component. In the last decades, several studies have been performed to explain how and why Si can improve the resistance and/or tolerance of higher plants to abiotic stresses. Si alleviates stress by stimulating the antioxidant system, complexing with toxic elements reducing their availability, confining toxic ions outside the root, interfering with the uptake and tissue distribution of heavy metals. Zinc (Zn) is an essential micronutrient for plants, being a cofactor and playing a fundamental role in the growth and development of higher plants; therefore, Zn deficiency induces severe symptoms, reducing crop yield. It has been shown that Si supply may ameliorate the damages induced by Zn toxicity in higher plants. This chapter will deal with many aspects that will be helpful to better understand the role that Si has in the alleviation of the symptoms caused by Zn deficiency and excess.

Availability of Si in the biosphere and uptake by plants

Si represents about 28% of the Earth's crust (Sommer et al.; 2006); nevertheless, it is not generally considered essential for higher plants (Epstein, 1999). The presence of Si in the biosphere is predicted to be limited to 0.03% (Fauteux et al.; 2005); several studies demonstrated that this element is largely accumulated in plants reaching values from 0.1 to 10% of dry weight (Hodson et al.; 2005). These levels appear to be higher than those observed for fundamental elements such as phosphorus (Epstein, 1994), supporting the importance of Si for plants.

Si present in soil waters is taken up by roots as silicic acid, $\text{Si}(\text{OH})_4$ (Exley, 1998). It has been suggested that Si movement is passive (Exley, 2015) and silicon channels have been identified in the past in several plant species (Ma et al., 2006, 2007; Yamaji et al., 2008).

In plants, the broad variation of Si levels in tissues is ascribed to changes in the uptake and transport (Jarvis, 1987; Epstein, 1994). Si is present in soil liquids in the form of silicic acid, $\text{Si}(\text{OH})_4$, generally ranging from 0.1 to 0.6 mM (Epstein, 1999).

In plant tissues, Si is accumulated as amorphous biogenic silica, SiO_2 (Matichencov and Bocharnikova 2001; Sauer et al., 2006). In soils, Si can be found as monosilicic and polysilicic acids, in their turn complexed with other organic or inorganic compounds (Cornelis et al., 2011). The presence of Si in soil solutions is able to change properties such as pH, cation compositions, water content and the availability of organic compounds (Sauer et al., 2006). Plants are generally able to absorb Si as $\text{Si}(\text{OH})_4$, when the pH of the soil is below 9 (Sommer et al., 2006). Si uptake by plants depends on the species and genotypes, thus Si content in plants can greatly vary (Epstein, 1994). In higher plants, three possible mechanisms of Si uptake have been defined: passive, active and rejective (Ma et al., 2004).

Monocotyledons, such as wheat, maize, rice and sugarcane, are able to absorb Si more efficiently than other species (Casey et al., 2003); in these plants, Si uptake is prominent (Casey et al., 2003; Rains et al., 2006), and Si channels have been described in many members of *Poaceae* (Ma et al., 2007; Chiba et al., 2009; Mitani et al., 2009). Particularly, rice plants accumulate considerable Si

amounts using two highly expressed genes. These code for Low silicon 1 (Lsi1), an aquaporin-like membrane protein (Ma et al., 2006) and Low silicon 2 (Lsi2), an anion transporter responsible for moving silicon out of the cell (Ma et al., 2007). The combined work of Lsi1 and Lsi2, differently located in the plasma membrane of root cells, defines the effective transport of Si through the Casparian strip and thus Si uptake (Ma et al., 2006-2007). It is therefore evident that Si transport is a balance of opposite influx and efflux (Mitani et al., 2009).

Passive absorption occurs in dicotyledonous: these plants absorb Si using the transpiration flow (Guntzer et al., 2012). Legumes represent a case-limit: these plants generally do not absorb nor accumulate a high amount of Si (Liang et al., 2005b).

Generally, more than 90% of Si taken up in the roots is moved to the aerial part of the plants and particularly to the leaves (Ma and Takahashi, 2002) by transpiration (Ma et al., 2006). The xylem sap contains disilicic acid, but mostly monosilicic acid (Casey et al., 2003; Mitani and Ma, 2005). Once in the shoots, Si is stored as amorphous $\text{SiO}_2 \cdot n\text{H}_2\text{O}$, near the transpiration sites (Ma et al., 2011). Commonly, Si is localized in the epidermis of shoots; in the roots, solid Si is present in the endodermis, accumulating in cell walls (Lux et al., 2003; Keller et al., 2015), where it is not further redistributed to other tissues (Hodson and Sangster, 1990). Formation, composition and localization of solid deposits of silica, known as phytoliths, can greatly vary among higher plant families, depending on environmental conditions and species (Li et al., 2014).

Positive effects of Si for plant growth and stress response

In old plant physiology manuals, Si was not generally considered essential for higher plants; nevertheless, Epstein and Bloom (2005) proposed that it should be added to the list of quasi-essential nutrient for plant health, since its valuable effects have been demonstrated for growth and development of many plants.

In the last years, several experiments demonstrated its protective role. Plants grown in a soil with Si supply resist lodging with an increased mechanical strength that enables them to keep an erect habit. This is due to an increased mechanical strength of plant cell walls conferred by the structural

presence of Si (Guerriero et al., 2016). Epidermal phytoliths also give to plants protection against dehydration due to excessive transpiration (Emadian and Newton, 1989).

Si supply results in favorable effects in various plants, both dicotyledons (e.g. *Cucumis sativus*) and monocotyledons, particularly under stress (Rogalla and Romheld, 2002; Ma and Takahashi, 2002; Shi et al., 2005a; Zhang et al., 2008; Vaculik et al., 2009; Feng et al., 2010; Collin et al., 2014). Si is able to protect plants from heavy metal toxicity, radiation, drought salinity, high temperatures (Bélanger et al., 2003; Côté-Beaulieu et al., 2009; Epstein, 2009).

Several heavy metals disturb photosynthetic efficiency and chloroplast structure. Silicon was proven to ameliorate the high Zn-induced chloroplast damage, protecting the photosynthetic pigments and increasing the expression of specific genes involved in photosynthetic mechanisms (Song et al., 2014) (Table 1).

Furthermore, a role for Si in the response to biotic stress has been reported: Si ameliorates the damages induced by different diseases, such as blast and powdery mildew, as well as pests, i.e., plant hopper and stem borer (Ma and Yamaji, 2006) (Figure 1).

Gene	Function	Normal Zn (0.15 μM)	High Zn concentration (2 mM)	Si effect vs. Normal Zn	Si effect vs High Zn concentration
<i>PsbY</i> Os08g02630	Mn-binding polypeptide of the Mn ₄ Ca cluster in O ₂ -evolving PSII	100%	65%	180%	150%
<i>PsaH</i> Os05g48630	10 kDa membrane protein at the surface of PSI - linked to PsaA and PsaD proteins	100%	90%	300%	160%
<i>PetC</i> Os07g37030	Protein binding the Rieske FeS center of the cytochrome <i>bc_f</i> complex	100%	60 %	410%	120%
<i>PetH</i> Os03g57120	Ferredoxin-NADP ⁺ reductase (FNR)	100%	185%	270%	225%
Os09g26810	Subunit of LHCII	100%	40%	170%	70%
Os04g38410	Subunit of LHCII	100%	70%	300%	130%

Table 1 – Effect of Si supplementation on photosynthetic gene expression in rice (*Oryza sativa*) plants grown under normal and high Zn conditions indicated as percentage of expression under normal Zn conditions (0.15 μ M). Modified from Song et al., (2014).

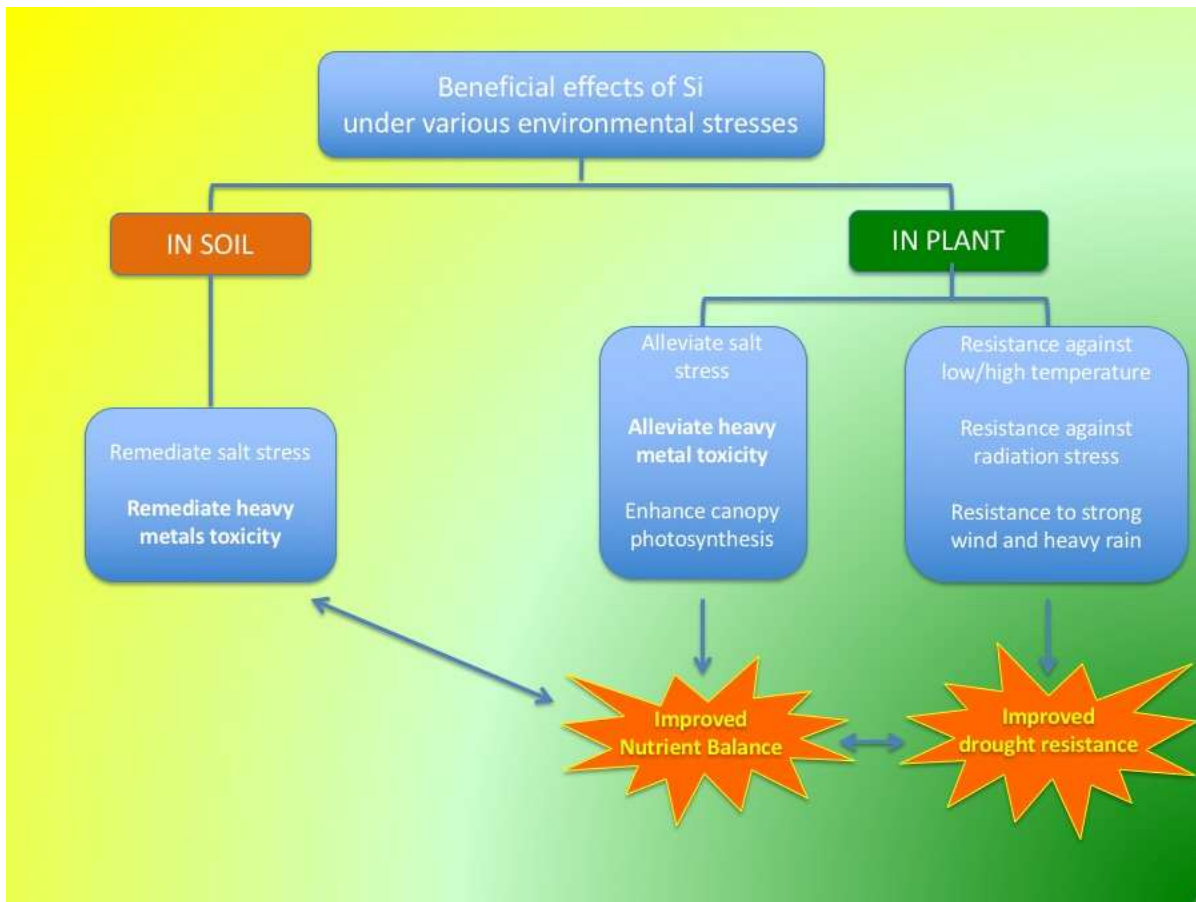


Fig. 1. Some beneficial effects of Si on plant growth and soil health in various conditions of environmental stress.

In the last decade, several progresses have been made, mostly in elucidating the role of Si in alleviating heavy metal stress (Li et al., 2008; Song et al., 2011; Dragisic Maksimovic et al., 2012). However, the knowledge on how Si can modulate nutrient shortage in plants is still limited. These beneficial roles are attributed to both soluble Si (monosilicic and polysilicic acids) and Si deposited in the various plant tissues as biogenic silica (SiO_2): Si deposits in tissues may function as a physical barrier to increase the rigidity and strength (Ma and Yamaji, 2006), while soluble Si stimulates defense reactions and enhances resistance against the diseases (Fauteux et al., 2005).

The emerging roles of Zn in basal plant metabolism and development

Heavy metals exert severe toxicological effects to living cells, from microorganisms to plants and animals; despite this, some are essential for correct growth and development of plants, thus they are considered micronutrients (Han et al., 2002). These elements include Zn that is considered both an essential micronutrient and a toxic metal in soils (Chaney 2010).

Zn is functionally absorbed in the form of Zn^{2+} ions. Zn is a structural stabilizer of a large number of proteins, e.g. transcription factors (such as B-box proteins, GATA factors, VOZ transcription factors) and metalloenzymes (such as carbonic anhydrase II, carboxypeptidase, superoxide dismutase, carbonate dehydratase, alcohol dehydrogenase, alkaline phosphatase) (Figueiredo et al.; 2012). Furthermore, Zn is an important cofactor in many enzymes, playing catalytic and co-catalytic roles; many of these Zn-containing enzymes play key steps in fundamental physiological processes, such as the synthesis and catabolism of biological molecules, from nucleic acids to lipids, from carbohydrates to proteins (Marschner, 1996; Pedas et al., 2009).

Furthermore, Zn limitation reduces the amount of proteins, starch, sucrose and severely affects flowering and seed development in sugar beet and maize (Singh et al., 2005).

Zn is essential in the protection of membranes against oxidative stress; it has been suggested that this element plays a critical role in the uptake of several nutrients and their homeostasis (Zhao et al., 2005).

In many parts of the world, agricultural soils undergo Zn shortage (Cakmak 2002) and this nutritional imbalance is a widespread – and certainly underestimated - micronutrient deficiency in crops (Cakmak et al., 1999). Although Zn is generally abundant in cultivated soils, its availability is often reduced and this deficiency is due to several factors that modify the micronutrient accessibility, acquisition or utilization by plants.

Predominantly, Zn deficiency occurs as a consequence of its low bioavailability, especially in alkaline soils, like saline, waterlogged and calcareous fields that severely restrict the availability of

this element for plants (Alloway 2008). Zn deficiency is an important factor that limits production by negatively affecting economically important crops in the world (Alloway, 2008).

Zn deficiency causes distinct symptoms such as reddish-brown or bronze tints, interveinal chlorosis in leaves, epinasty, shortening of internodal distance, curled and smaller leaves (Marschner, 1995).

Positive roles of Si to counteract Zn deficiency in plants

Plants remobilize nutrients stored in vegetative tissues during senescence or under nutrient deficiency, moving these to seeds, fruits or young leaves (Waters et al., 2009). The main storage sites are cell vacuoles, and particularly in shoots, a high concentration of metals can be stored as organic acid complexes (Sinclair and Krämer, 2012).

Several studies confirm that Si plays beneficial roles in alleviating Zn stress, and the main Si influence is given by the evident improvement of Zn distribution in the plant (Gu et al., 2011, 2012; Bityuskii et al., 2014). Gu et al., (2011) demonstrated that Zn and Si are similarly located in plants, mostly in the endodermis of roots, as precipitated Zn silicates. These compounds can be slowly degraded to SiO₂, while Zn remains stored in vacuoles under a still unknown form (Neumann and zur Nieden, 2001).

As consequence, on cell walls where silicates precipitate, Zn²⁺ binding sites increase, thus enhancing Zn²⁺ absorption (Wang et al., 2000). This phenomenon may act as a protective mechanism of detoxification, since it keeps Zn²⁺ at the same time adsorbed, but away from more sensitive organs (da Cunha and do Nascimento, 2009; Shi et al., 2005b; Hodson and Sangster, 1999).

Moreover, Hernandez-Apaolaza (2014) suggested that deposits of Zn stored in the roots could be mobilized under Zn deprivation, if Si is present; as a consequence, Si would be able to mobilize Zn, enhancing its allocation and utilization by the plant through a mechanism not yet studied. Zn and Si share the same localization in shoots as well, and this may be caused by a similar transport through the xylem (Gu et al., 2012). Thus, under Zn deficiency, nutrients are remobilized and transported

through the phloem, increasing Zn pools in seeds and fruits and enhancing Zn availability (Bityutskii et al., 2014).

Gonzalo et al, (2013) postulated that higher doses of Si would not be beneficial for plants, since high Si could induce an equilibrium displacement for large Zn amounts removed from soil solutions; therefore, if Si was supplied at high concentration, the nutrient solution would be saturated with amorphous silica that may chelate Zn, avoiding root uptake.

The excess of Zn and possible use of Si to ameliorate Zn stress

Despite the widespread deficiency of the micronutrient, in the last decades the levels of Zn in soils have rapidly increased due to a wide use of the nutrient in industry, mines, smelting and waste (Luo et al., 2000; Zhao et al., 2003, Song et al., 2011).

Above a certain optimum concentration (300mg kg^{-1}), Zn converts into a toxic element to plant cells (Ehsan et al., 2013). An excessive Zn content affects growth, morphology and metabolism of cultivated plants, thus limiting crop yields (Cuypers et al., 1999).

Zn is accumulated in root vacuoles, bound to organic acids. When in excess, Zn results in reduced biomass, possibly caused by the inhibition of meristematic mitoses, and reduced elongation of the root cells. It is noteworthy that Zn easily binds to cell wall components, such as cellulose, pectins, hemicellulose and structural proteins, thus altering the regular development of the root (Krzyszowska; 2011).

Furthermore, Zn excess causes an unbalance in the micronutrient content in plant cells, particularly affecting Mn, Fe and Cu levels. It has been suggested that these effects can be – at least in good portion – caused by the diversion of energy consumed by stress-responding systems to counteract the accumulation of Zn (Glińska et al., 2016).

Zn excess negatively affects mineral uptake and translocation by modifying the nutrient homeostasis. As result, a deficiency in essential ions, such as Mg, Fe and P was observed; the final

consequence is the limitation or damage of essential metabolic processes, like photosynthesis, transpiration and other metabolic pathways (Ali et al., 2013b; Abbas et al., 2009).

Plants show different susceptibility to Zn exposure and carry out various mechanisms of tolerance to this stress (Zorrig et al., 2010). In condition of Zn toxicity, plants can produce some nitrogenous low molecular weight compounds, such as proline, glycine betaine and γ -aminobutyric acid, involved in the xylem nitrogen transport and in metal detoxification. These osmolytes may represent a signal of tolerance to toxicity or, when their levels increase in susceptible plants, these molecules constitute a symptom of Zn stress (Paradisone et al., 2015).

Figure 2 shows some mechanisms of metal toxicity alleviation mediated by Si in plants, as suggested in recent studies.

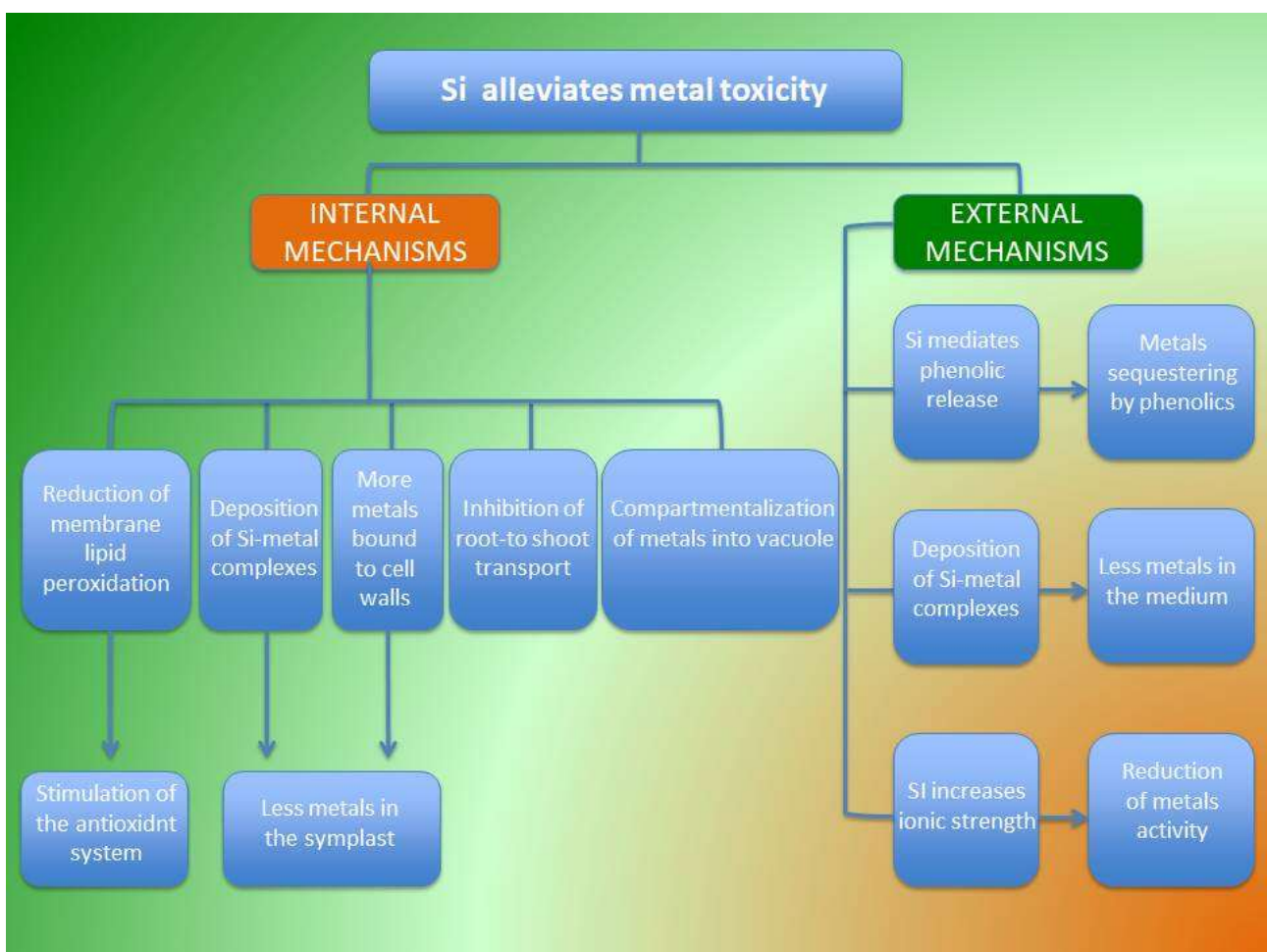


Fig. 2. Mechanisms of Si ameliorating metal toxicity (modified from Liang et al., 2005).

The beneficial effects of Si could act both externally and/or induce specific inner plant processes (Cocker et al., 1998; Wang et al., 2004). The benefits of Si on Zn excess mainly consist in a decrease of metal phytoavailability in the growth media induced by increasing pH (minerals containing Si are generally alkaline) and the formation of metal silicates, which are biologically inert, in the soils (Cocker et al.; 1998; Ma et al., 1997).

Si treatment reduces Zn toxicity during seedling growth, with a consequent increase of plant biomass, when sufficient silicate is supplied (Gu et al., 2011). Interestingly, Zn distribution in different tissues changes with the presence of Si in soil (Gu et al., 2012). Song et al. (2011) documented that Si supply reduced the translocation of Zn from roots to shoots. Si supply induces polysilicate formation in the cell wall changing the binding capacity for metals and particularly Zn, thus reinforcing the cell walls (Brasser et al., 2006). In addition, the integration of silicate compounds in the root cell walls improves the number of binding sites for Zn ions, thus increasing the amount of Zn in the cell wall matrix (Currie and Perry 2007). It was shown that metal sequestration by cell walls of structural tissues can play an important role in the detoxification process, preventing toxic cations from reaching more sensitive cells, such as the mesophyll cells (Hodson and Sangster 1999; Neumann et al., 1997).

As described before, Zn distribution is affected by silicate addition and Zn and Si distributions are similar, with a preference for the root endodermis: silicate(s) increase the sequestration of cations in the cell wall, due to the metal ion binding sites and resulting in a combination between silicate and metals (Wang et al., 2000). Thus, Si supply is able to decrease Zn levels in the xylem sap, possibly by forming insoluble complexes between Si and Zn; moreover, the deposition of these precipitates might partially restrain the loading of Zn in the xylem, further reducing Zn transport (da Cunha and do Nascimento, 2009).

Finally, in this chapter the positive consequences of Si supply on the development of plants grown under both deficient and toxic Zn levels have been summarized. Nonetheless, further studies are

necessary to better depict the molecular and biochemical mechanisms inducing these effects, in order to ameliorate our understanding of the effects of Si on Zn stress.

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Chapter 3

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Roles of some nitrogenous compounds protectors in the resistance to zinc toxicity in *Lactuca sativa* cv. Phillipus, and *Brassica oleracea* cv. Bronco

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Additional Key words

Proline; glycine betaine; γ -aminobutyric acid; Zn-toxicity; stress tolerance.

Abbreviations

Pro, proline; P5CS, Δ^1 -pyrroline-5-carboxylate synthetase; OAT, ornithine- δ -aminotransferase; PDH, proline dehydrogenase; GB, glycine betaine; BADH, betaine-aldehyde dehydrogenase; ROS, reactive oxygen species; GABA, γ -aminobutyric acid; GAD, glutamate decarboxylase; DAO, diamine oxidase; PAO, polyamine oxidase.

Chapter 3

Roles of some nitrogenous compounds protectors in the resistance to zinc toxicity in *Lactuca sativa* cv. Phillipus, and *Brassica oleracea* cv. Bronco

Abstract

Zinc (Zn) pollution in the soils represents a major problem for crop production worldwide. In the present work, two horticultural plants exhibiting different tolerance to Zn, *Lactuca sativa* cv. Phillipus, and *Brassica oleracea* cv. Bronco, were exposed to Zn in order to evaluate the contribution of compatible osmolytes as proline (Pro), glycine betaine (GB) and γ -aminobutyric acid (GABA), in the mechanism(s) of tolerance to Zn stress.

This study confirms the higher susceptibility of *L. sativa* to Zn stress: lettuce plants experienced a strong reduction in biomass; in the meanwhile, the levels of Pro and GB increased. These results would suggest that in *L. sativa* the increase of Pro and GB does not represent a mechanism of resistance to toxicity, but it is likely a symptom of Zn-stress.

Conversely, in *B. oleracea*, a slight decrease in Pro levels, mainly catalysed by degradation through proline dehydrogenase (PDH), was observed; a similar behaviour affected GB levels. On the other hand, GABA synthesis was slightly, but significantly, increased. The presence of high levels of GABA in Zn-stressed *B. oleracea* would suggest that reactive oxygen species (ROS) detoxification could be essential to improve the resistance to toxicity under metal stress conditions.

Introduction

Metal toxicity is one of the major environmental threats to the ecosystems, because of its continuous increase, caused by both natural sources and human activities; among the many metals, Zinc (Zn) represents one of the most widespread pollutants in the environment.

It is noteworthy that Zn is an essential micronutrient required as cofactor in many enzymatic reactions and it is involved in various physiological and metabolic processes. Zn has also a stabilizing and protective effect on biomembranes during oxidative damage, by a preservation of plasma membrane integrity and permeability (Bettger and O'Dell 1981). Moreover, Zn alleviates other metal-induced oxidative stress (Upadhyay and Panda 2010). It is generally available in the soil for plant uptake as zinc sulphate, chelated and / or linked zinc (Thounaojam et al. 2012).

Zinc emissions from industrial activities are the main source of pollution in the crops worldwide. Zn accumulates in plant tissues, reaching toxic levels, thus provoking physiological alterations and growth inhibition. These effects are caused, among other processes, by changes in carbohydrate metabolism (Foy et al. 1978), a decrease in the content of essential nutrients such as Fe, Cu, and Mn (Sargodoy et al. 2011), an oxidative damage and the alteration of photosynthetic activity (Vassilev et al. 2007).

There is broad variability in the susceptibility and tolerance to exposure to Zn, as well as in the tolerance mechanisms in plants (Zorrig et al. 2010). It is well known that, under metal stress, plants are able to synthesise low-molecular-weight compounds involved in the long-distance transport in the xylem, and in the heavy metals detoxification.

Commonly, these compounds are compatible organic solutes, usually harmless at high cellular concentrations (Sharma et al. 1998; Chen et al. 2007). These molecules can protect plants from stress in different ways, including cellular osmotic adjustment, detoxification of reactive oxygen species (ROS), protection of membrane integrity, and stabilization of proteins (Chen et al. 2007). Furthermore, some of them preserve cellular components from dehydration, thus they are commonly recognised as osmoprotectants; these solutes include proline (Pro), and quaternary

ammonium compounds, such as glycine betaine (GB), and γ -aminobutyric acid (GABA) (Rhodes and Hanson 1993).

Levels of Pro are able to control the expression of genes coding of enzymes involved in stress response and supply of reductants. It has been demonstrated that the increase in Pro levels is linked to glucose-6-phosphate dehydrogenase activities in salt-stressed barley (Cardi et al. 2015); similarly, Zn-induced inhibition of glucose-6-phosphate dehydrogenase (G6PDH) is reduced by the increase of Pro levels (Sharma et al. 1998).

GB plays different protective roles: i) the stabilization of the structures of enzymes and proteins; ii) the reduction of ROS levels under stress; iii) the preservation the stability of membranes under non-physiological conditions (Chen and Murata 2002). In addition to these direct protective roles, GB levels play a part in the signal transduction during the environmental stress: both the exogenous supply of GB, and the genetically engineered biosynthesis of GB increase the tolerance of plants to abiotic stress (Chen and Murata 2002). It has been demonstrated that, in transgenic plants under water stress, GB reduces ROS accumulation, increasing the resistance to drought (Kathuria et al. 2009).

GABA has pivotal important roles in the nitrogen metabolism (Fait et al. 2007) and in the response to different stressors (Bor et al. 2009). Intriguingly, the time-scale accumulation of GABA in response to environmental stress follows a similar pattern in different species, which raises questions on its possible role as a unique signal for stress in plants (Kinnersley and Turano 2000).

It has been hypothesised that GABA could be involved in the resistance to heavy metals; its degradation could limit the accumulation of ROS under oxidative stress conditions, by inhibiting enzymes of the TCA cycle and lowering the levels of ROS, by transfer reactive intermediates from cytosol to mitochondria (Fait et al. 2007).

Many studies have previously highlighted several differences between the capability to store osmo-protectants, and the tolerance mechanism(s) in different plants under metal-stress.

In this work, a comparative study has been made on two species of great agricultural interest, *L. sativa* (lettuce) and *B. oleracea* cv. Bronco (white cabbage), where the effects Zn toxicity on the levels of some nitrogenous compounds, and their metabolism, have been investigated. The possible key role of these pathways in establishing a tolerance to Zn toxicity in plant will be discussed. These data will be useful to identify possible osmolytes and/or enzymatic activities able to confer tolerance to Zn effects in plants, in order to select varieties suitable for phytoremediation of Zn-contaminated soils.

Materials and methods

Growth of plants and experimental design

Seeds of *Lactuca sativa* cv. Phillipus, and *Brassica oleracea* cv. Bronco were germinated and grown for 35 days in cell flats of 3 x 3 x 10 cm filled with a perlite mixture substrate. The flats were placed on benches in an experimental greenhouse located in Spain (Granada, Motril, Saliplant S.L.). After 35 days, seedlings were transferred to a growth chamber under controlled environmental conditions, with relative humidity of 50%, day/night temperature of 25/15°C, a photoperiod of 16/8 h under a photosynthetic photon flux density (PPFD) of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (measured at the top of the seedlings with a 190 SB quantum sensor, LI-COR Inc., Lincoln, Nebraska, USA). Plants were grown in hydroponic culture in lightweight polypropylene trays (60 cm diameter top, bottom diameter 60 cm and 7 cm in height) with a volume of 3 litres. Throughout the experiment the plants were treated with a growth solution composed as follows: 4 mM KNO_3 , 3 mM $\text{Ca}(\text{NO}_3)_2 \cdot 4 \text{H}_2\text{O}$, 2 mM $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$, 6 mM KH_2PO_4 , 1 mM $\text{NaH}_2\text{PO}_4 \cdot 2 \text{H}_2\text{O}$, 2 μM $\text{MnCl}_2 \cdot 4 \text{H}_2\text{O}$, 10 μM $\text{ZnSO}_4 \cdot 7 \text{H}_2\text{O}$, 0.25 μM $\text{CuSO}_4 \cdot 5 \text{H}_2\text{O}$, 0.1 μM $\text{Na}_2\text{MoO}_4 \cdot 2 \text{H}_2\text{O}$, 5 ppm Fe-EDTA (Sequestrene; 138 FeG100, Syngenta) and 10 μM H_3BO_3 . The nutrient solution was checked for a pH of 5.5-6.0, and changed every three days. Treatments were initiated 35 days after germination and maintained for 21 days. Control plants were grown in the basal nutrient solution, while stressed plants were supplemented with 0.5 mM of Zn as ZnSO_4 . The experimental design consisted of randomized complete block with four treatments (*L. sativa* - control, *B. oleracea* - control, *L. sativa* - 0.5 mM Zn, *B. oleracea* - 0.5 mM Zn), eight plants per treatment in three independent biological replicates (Fresh Weights in Supplemental Table 1).

Zn concentration

For the determination of Zn concentration, a sample of 30 mg dry material was subjected to a process of mineralization by the method of Wolf (1982). The detailed procedure has been described elsewhere (Barrameda-Medina et al. 2014).

Measurements of proline, glycine betaine, and γ -aminobutyric acid

For the determination of Pro, leaves were homogenized in 5 ml of 96% ethanol. Insoluble fraction was washed with 70% ethanol. The extract was centrifuged at 3500 g for 10 min and the supernatant stored at 4°C (Irigoyen et al. 1992); 1 ml aliquot of the supernatant was taken and, after adding reactive ninhydrin acid reagent (ninhydrin, 6M phosphoric acid, 60% glacial acetic acid) and 2.5 ml of 99% glacial acetic acid, was placed in a water bath at 100°C; after 45 min, the tubes were cooled on ice, and 5 ml of benzene were added; after 5-10 min the absorbance of the organic phase was measured at 515 nm.

Glycine betaine (GB) was determined as described by Grive and Gratton (1983): GB was extracted from 200 mg of dry plant material in 10 ml of distilled water, gently shaking for 24 hours. The extract was filtered and 2 ml of 2 N H₂SO₄ were added. Then the solution was incubated for 16 h at 4°C and centrifuged at 9000 g for 15 min at 0°C. The pellet was suspended in 1.2 dichloroethane; after 2 hours, the GB content was measured by reading the absorbance at 365 nm and quantified using a standard curve of GB.

γ -aminobutyric acid (GABA) was measured by the method of Zhang and Bown (1997): frozen leaves were ground in liquid nitrogen, and 100 mg of the powdered tissue were extracted in 0.1 ml methanol for 10 min at room temperature. The slurry was vacuum dried with a Vacufuge concentrator 5301 (Eppendorf, Hamburg, Germany). The dried powder was dissolved in 1 ml 70 mM lanthanum chloride, shaken for 15 min, and then centrifuged at 13000 g for 5 min. The clear supernatant was transferred to a new tube, and 0.8 ml of supernatant were mixed with 0.16 ml 1 M potassium hydroxide, shaken for 5 min, and centrifuged at 13000 g for 5 min. The supernatant was used for the determination of GABA.

GABA content was measured using GABase (Sigma, St. Louis, MO, USA): 1 ml assay mixture contained 0.6 mM NADP⁺, 0.1 unit GABase, 0.1 M potassium pyrophosphate buffer (pH 8.6), 1 mM α -ketoglutarate and 550 μ l sample. The reduction of NADP⁺ to NADPH was monitored at 340 nm in a spectrophotometer after 10 min incubation at room temperature.

Extraction and determination of enzymatic activities

Δ^1 -pyrroline-5-carboxylate synthetase (P5CS) extraction was carried out according to Sumithra et al. (2006). Leaves (100 mg) were homogenized in the extraction buffer [100 mM Tris-HCl (pH 7.5), 10 mM β -mercapto-ethanol, 10 mM $MgCl_2$ and 1 mM phenyl-methyl-sulfonyl fluoride (PMSF)], and then centrifuged at 10000 g for 15 min; the clear supernatant was used for enzyme assays. P5CS activity was measured as described by Charest and Phan (1990): the reaction mixture contained 100 mM Tris-HCl (pH 7.2), 25 mM $MgCl_2$, 0.4 mM NADPH, 5 mM ATP and extract. The reaction was initiated by the addition of 75 mM sodium glutamate and the activity was measured as the rate of consumption of NADPH monitored as absorbance decrease at 340 nm.

Ornithine- δ -aminotransferase (OAT) and proline dehydrogenase (PDH) were extracted from leaves (100 mg) homogenized in 100 mM potassium-phosphate buffer (pH 7.8). The homogenate was filtered and centrifuged at 12000 g for 20 min (4°C). OAT was assayed according to Charest and Phan (1990) in 0.2 M Tris-KOH buffer (pH 8.0) containing 5 mM ornithine, 10 mM α -ketoglutarate and 0.25 mM NADH. The decrease in absorbance of NADH was monitored at 340 nm for 1 min after initiating the reaction with the addition of the extract. PDH activity was assayed by the reduction of NAD^+ at 340 nm. The reaction mixture contained 0.15 M Na_2CO_3 -HCl buffer (pH 10.3) containing 2.67 mM L-proline and 10 mM NAD^+ .

Protein extraction and NAD^+ -dependent betaine aldehyde dehydrogenase (BADH) activity measurements were made as in Tang (1999). One gram of fresh leaf tissue was ground in a mortar on ice with 2 ml of cold extraction buffer [50 mM Hepes/KOH, pH 8.0, 5 mM dithiothreitol]. Leaf debris were removed by centrifugation at 14500 g for 10 min at 4°C. The BADH activity was determined measuring at 340 nm at 37°C the rate of formation of NADPH in a 3 ml mixture consisting of 50 mM HEPES (4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid) - KOH buffer (pH 8.0), 5 mM DTT, 1 mM EDTA, 1 mM betaine aldehyde, 1 mM NAD^+ and extract. Each assay was made in duplicate.

Cytosolic glutamate decarboxylase (GAD) was extracted from plant material, as described previously, in a buffer containing 0.1 M Tris-HCl (pH 9.1), 10% (v/v) glycerol, 1 mM dithiothreitol (DTT), 5 mM ethylene-diamine-tetra-acetic acid (EDTA), 0.5 mM pyridoxal phosphate (PLP) and 1 mM phenyl-methyl-sulfonyl-fluoride (PMSF). Fresh tissue was added to the pre-cooled extraction buffer at a ratio of 1:3 (w/v) and homogenized at 4°C. The homogenate was filtered through one Miracloth layer, then centrifuged at 24500 g at 4°C for 30 min. The supernatant was used for GAD activity determination using the method of Bartyzel et al. (2003): the enzyme rate was assayed by incubating the extract at 30°C for 60 min in 400 µl assay mixture containing 0.1 M potassium phosphate buffer (pH 5.8), 40 µM PLP and 3 mM L-glutamate. The reaction was stopped by adding 0.1 ml of 0.5 mM HCl; the amount of GABA in the samples was determined as previously described.

Diamine oxidase (DAO) and polyamine oxidase (PAO) activities were determined by measuring H₂O₂ production, as described by Su et al. (2005), with few modifications. Plant material (700 mg) was homogenized in 100 mM potassium-phosphate buffer (pH 6.5); the homogenate was centrifuged at 10000 g for 20 min at 4°C and the supernatant used for the assays. The reaction mixture contained 2.5 ml of potassium-phosphate buffer (100 mM, pH 6.5), 0.2 ml of 4-aminoantipyrine/N,N-dimethyl-aniline reaction solution, 0.1 ml of horseradish peroxidase (250 U/mL), and 0.2 ml of the enzyme extract. The reaction was initiated by the addition of 0.15 ml of 20 mM putrescine for DAO determination and 20 mM spermidine plus spermine for PAO determination. One unit of enzyme activity corresponded to a change in 0.001 absorbance unit at 555 nm per min under the condition described.

Statistical Analysis

Data were analysed by ANOVA test; differences between means were compared by Fisher's Least Significant Difference (LSD) ($p > 0.05$).

Results and Discussion

Zinc (Zn) is a micronutrient playing an essential role in many physiological and biochemical processes; when exposed to an excess of Zn, plants accumulate this element in their tissues, where it can reach toxic concentrations, thus provoking metabolic alterations and inhibition of growth (Gisbert et al. 2006).

In natural environments, Zn occurs in water, air and soil, but its concentrations are increasing, largely due to anthropic activities. Plants are not able to manage excess of Zn by their physiological uptake systems, thus resulting in a serious threat to agricultural production (Vassilev et al. 2007).

L. sativa and *B. oleracea* accumulated Zn in the roots when supplied with 0.5 mM Zn, up to 35-45 mg g⁻¹ DW (Table 1); a corresponding decrease in root biomass (about 35%) was observed in both species.

In the leaves, differences were observed between the two species: in *L. sativa* the application of 0.5 mM Zn caused a 2.7-fold accumulation of Zn, with a related reduction in biomass (-33%); in *B. oleracea* a 6-fold Zn accumulation was detected, without a significant change in biomass (Table 1), although Zn levels reached values considered as toxic (Marschner, 1995).

It has been suggested that exposition to Zn caused its accumulation both in leaves and in roots (Ozdener and Aydin 2010), due to compartmentation (Gisbert et al. 2006) or to the adsorption in the apoplast (Küpper et al. 2000).

Our results suggest that the effects of toxic Zn levels on the biomass are particularly harmful in the roots. On the other hand, changes in the leaf biomass are mutable, depending on the susceptibility to stress of the different species: *Brassicaceae* seem unresponsive to Zn in the leaves (Gisbert et al. 2006), while *Crassulaceae*, such as *Sedum alfredii*, showed an increase in biomass at 0.5 mM Zn, without any toxic effects in the leaves (Yang et al. 2006). Our results clearly suggest that under exposure to toxic conditions of Zn, *B. oleracea* accumulates Zn in both roots and leaves, but toxic effects are evident only in the roots, while *L. sativa* showed a marked decrease in both roots and leaves biomass.

Pro is possibly the most known osmolyte produced by plant cells in response to abiotic stress. In plants, Pro is synthesised from either glutamate or ornithine, by Δ^1 -pyrroline-5-carboxylate synthetase (P5CS), or ornithine- δ -aminotransferase (OAT), respectively. The accumulation of Pro in the response to different types of stress is directly related to enzyme activities and/or transcriptional activation of the genes encoding these enzymes, thus inducing an improved capability to survive under stress conditions (Strizhov et al. 1997).

Pro levels notably increased upon treatment with 0.5 mM Zn in *L. sativa* (+44%) and are correlated to an increase of OAT (+40%). In contrast, Zn induced a slight reduction of Pro levels in *B. oleracea* with respect to control plants (Table 2); correspondingly, Zn-treated *B. oleracea* did not exhibit major changes in P5CS and OAT activities.

L. sativa is more sensitive than *B. oleracea* to Zn stress, thus our results do not support the hypothesis that proline accumulation could be associated with an increased tolerance against heavy-metal stress, as previously proposed (Tripathi and Gaur 2004). In *L. sativa* Pro increase would represent a symptom of Zn stress, rather than a mechanism of resistance against Zn toxicity; this agrees with the hypothesis of Schat and Vooijs (1997), who suggested that metal-induced Pro accumulation is a consequence, and not a cause, of metal tolerance in plants.

Furthermore, the increase in Pro levels upon metal stress can be possibly caused by a decrease in Pro degradation process (Kavi Kishor et al. 2005), due to mitochondrial proline dehydrogenase reaction (PDH) (Lin and Kao 2007).

Table 1 Levels of Zn in roots, leaves and changes in biomass in *L. sativa* and *B. oleracea* in control plants, and plants exposed to 0.5 mM Zn.

Cultivar/Treatments	[Zn] root (mg g ⁻¹ DW)	Root Biomass (g DW/plant)	[Zn] shoot (µg g ⁻¹ DW)	Leaf Biomass (g DW/plant)
<i>Lactuca sativa</i> cv. Philipus				
Control	0.586 ± 0.0687	0.135 ± 0.003	80.78 ± 4.39	2.11 ± 0.19
0.5 mM Zn	34.949 ± 0.376	0.042 ± 0.010	218.4 ± 35.7	1.43 ± 0.04
<i>p</i> -value	***	***	**	*
LSD _{0.05}	10.441	0.03	83.02	0.53
<i>Brassica oleracea</i> cv. Bronco				
Control	0.895 ± 0.104	0.083 ± 0.003	61.43 ± 0.66	0.70 ± 0.06
0.5 mM Zn	42.541 ± 0.234	0.033 ± 0.006	382.4 ± 60.6	0.61 ± 0.08
<i>p</i> -value	***	**	***	NS
LSD _{0.05}	0.863	0.02	139.86	0.29

Values are means (n=9) ± standard error. Legend: DW: dry weight; LSD_{0.05}= Fisher's Least Significant Difference (LSD) (p> 0.05); *p*-values: *, <0,05; **, <0,01; ***, < 0.001. NS: Not Significant.

Table 2 Proline levels, and activities of enzymes of proline metabolism in *L. sativa* and *B. oleracea* control plants, and plants exposed to 0.5 mM Zn. Pro, proline; P5CS, Δ 1-pyrroline-5-carboxylate synthetase; OAT, ornitine- δ -aminotransferase; PDH, proline dehydrogenase.

Cultivar/Treatments	Pro ($\mu\text{mol g}^{-1}\text{FW}$)	P5CS ($\Delta\text{Abs min}^{-1} \text{mg prot}^{-1}$)	OAT ($\Delta\text{Abs min}^{-1} \text{mg prot}^{-1}$)	PDH ($\Delta\text{Abs min}^{-1} \text{mg prot}^{-1}$)
<i>Lactuca sativa</i> cv. Philipus				
Control	0.27 \pm 0.03	0.084 \pm 0.007	0.10 \pm 0.008	0.10 \pm 0.008
0.5 mM Zn	0.39 \pm 0.01	0.081 \pm 0.005	0.14 \pm 0.009	0.08 \pm 0.006
<i>p</i> -value	**	NS	**	NS
LSD _{0.05}	0.07	0.02	0.02	0.05
<i>Brassica oleracea</i> cv Bronco				
Control	0.42 \pm 0.02	0.045 \pm 0.012	0.05 \pm 0.003	0.08 \pm 0.005
0.5 mM Zn	0.37 \pm 0.01	0.050 \pm 0.006	0.05 \pm 0.002	0.15 \pm 0.009
<i>p</i> -value	*	NS	NS	**
LSD _{0.05}	0.03	0.03	0.02	0.03

Values are means (n=9) \pm standard error. Legend: FW: Fresh weight; LSD_{0.05}= Fisher's Least Significant Difference (LSD) ($p > 0.05$); *p*-values: *, <0,05; **, <0,01; ***, < 0.001. NS: Not Significant.

In *L. sativa*, PDH activity remained substantially unaltered in Zn-treated plants, thus confirming that the synthesis (OAT), and not the degradation (PDH), is the cause of the increased Pro levels under Zn stress; similar results were obtained in Zn-exposed wheat seedlings, where Zn-induced Pro accumulation was associated to a prompt rise of OAT activity, and an inhibition of PDH (Li et al. 2013).

In contrast, the improved Zn tolerance in *B. oleracea* is supported by a two-fold increase in PDH activity, thus decreasing Pro levels.

Glycyl-betaine (GB) accumulates in response to heavy-metal stress in plants; GB is synthesised from choline by a two-step oxidation reaction catalysed by choline monooxygenase (CMO) and NAD⁺-dependent betaine aldehyde dehydrogenase (BADH) to GB (Chen and Murata 2002); therefore, changes in GB levels could be related to variations in BADH activity.

L. sativa plants treated with 0.5 mM Zn showed higher levels of GB (+ 80%) as previously reported for other plants upon abiotic stress (Cardi et al. 2015). Interestingly, a corresponding +35% increase in BADH activity with respect to untreated plants was observed. *L. sativa* is sensitive to Zn stress, thus our data contrast with the hypothesis that GB accumulation represents a symptom of an enhanced tolerance to abiotic stress (Sakamoto and Murata 2000).

On the contrary, in *B. oleracea*, Zn exposure led to a reduction of GB levels at 50% of their initial value, leaving BADH activity substantially unaltered (Table 3).

It has been suggested that low levels of GB may preserve membranes and photosynthetic apparatus upon abiotic stress (Sakamoto and Murata 2000; Yang et al. 2006); salt stress triggers an increase in GB levels in barley (Cardi et al., 2015), and higher levels of GB were observed in salt-sensitive barley cultivars with respect to salt-tolerant cultivar (Chen et al. 2007). These authors hypothesised that a low GB

concentration in salt-tolerant plants is required to alleviate stress toxic effects, while the accumulation in salt-sensitive plants could represent a symptom of stress damage; anyway, the relationship between GB accumulation and enhanced stress tolerance is possibly specie-specific (Ashraf and Foolad 2007).

In plants, GABA, a non-protein amino acid, plays an important role as osmoprotectant (Rhodes and Hanson 1993); it is mainly synthesised through “the GABA shunt”. This pathway originated from cytosolic glutamate decarboxylase (GAD) reaction, (Fait et al. 2007); GABA is produced from polyamines degradation by the coordinated action of diamine oxidase (DAO) and polyamine oxidase (PAO) (Alcazar et al. 2010).

Table 3 Levels of glycine betaine, and activities of betaine aldehyde dehydrogenase (BADH) in *L. sativa* and *B. oleracea* control plants, and plants exposed to 0.5 mM Zn.

Cultivar/Treatments	Glycine betaine (nmol g ⁻¹ FW)	BADH (ΔAbs min ⁻¹ mg prot ⁻¹)
<i>Lactuca sativa</i> cv. Philipus		
Control	1.83± 0.15	0.20 ± 0.02
0.5 mM Zn	3.33 ± 0.32	0.27 ± 0.02
<i>p</i> -value	**	**
LSD _{0.05}	0.99	0.03
<i>Brassica oleracea</i> cv. Bronco		
Control	1.90 ± 0.12	0.05 ± 0.007
0.5 mM Zn	0.99 ± 0.09	0.03 ± 0.002
<i>p</i> -value	*	NS
LSD _{0.05}	0.67	0.04

Values are means (n=9) ± standard error. Legend: FW: Fresh weight; LSD_{0.05}= Fisher's Least Significant Difference (LSD) (p> 0.05); *p*-values: *, <0,05; **, <0,01; ***, < 0.001. NS: Not Significant.

In Zn-treated *L. sativa* GABA levels remained unaltered, while *B. oleracea* showed a small (+10%), but significant accumulation of GABA, possibly related to an increased synthesis (Table 4).

Actually, Zn-treated *B. oleracea* showed a significant 60% increase of GAD activity; differently, GAD activity remained unchanged after Zn exposure in *L. sativa* (Table 4). Concomitantly, both *L. sativa* and *B. oleracea* did not show any significant changes in PAO and DAO activities in response to Zn supply (Table 4).

These results would suggest that in *L. sativa* GABA does not play a key role in response to Zn stress.

Furthermore, it should be considered that GABA shunt plays a role in the provision of succinate and NADH to the respiratory chain; thus, it has been postulated that GABA levels may play a role to contrast ROS accumulation upon oxidative stress (Bouchè and Fromm 2004). Therefore, it can be proposed that in *B. oleracea* GABA may play a role to avoid the effects of Zn.

Table 4 Levels of γ -aminobutyric acid (GABA), and related enzymes, in *L. sativa* and *B. oleracea* control plants, and plants exposed to 0.5 mM Zn. GAD, glutamate decarboxylate; DAO, diamine oxidase; PAO, polyamine oxidase.

Cultivar/Treatments	GABA (nmol g ⁻¹ FW)	GAD (Δ Abs min ⁻¹ mg prot ⁻¹)	PAO (Unit mg ⁻¹ protein min ⁻¹)	DAO (Unit mg ⁻¹ protein min ⁻¹)
<i>Lactuca sativa</i> cv. Philipus				
Control	0.98 \pm 0.09	0.11 \pm 0.02	6.69 \pm 0.68	8.81 \pm 0.95
0.5 mM Zn	1.04 \pm 0.08	0.09 \pm 0.01	6.28 \pm 0.51	10.82 \pm 0.75
<i>p</i> -value	NS	NS	NS	NS
LSD _{0.05}	0.14	0.05	1.91	2.72
<i>Brassica oleracea</i> cv. Bronco				
Control	1.40 \pm 0.07	0.08 \pm 0.01	3.16 \pm 0.45	7.05 \pm 0.82
0.5 mM Zn	1.53 \pm 0.06	0.13 \pm 0.01	2.01 \pm 0.32	7.14 \pm 0.52
<i>p</i> -value	*	**	NS	NS
LSD _{0.05}	0.11	0.03	1.64	2.31

Values are means (n=9) \pm standard error. Legend: FW: Fresh weight; LSD_{0.05}= Fisher's Least Significant Difference (LSD) (p> 0.05); p-values:

*, <0,05; **, <0,01; ***, < 0.001. NS: Not Significant.

In conclusion, in *L. sativa* Zn toxicity is able to induce an increase in the levels of both Pro and GB; since these plants showed a significant decrease in their biomass, it is assumed that these two compounds do not represent a signal of resistance to toxicity, but probably a symptom of Zn-stress.

Differently, in *B. oleracea* Zn stress caused an increment of Pro degradation, catalysed by PDH activity, and an increase in GABA levels. These two processes resulted in an improved Zn tolerance in *B. oleracea* and suggest that ROS detoxification under Zn-stress can be essential to provide the metal-tolerance. Therefore, these data support our previous work, demonstrating that the homeostasis of GSH and its role in oxidative metabolism are essential to improve resistance of *B. oleracea* to Zn toxicity.

Authors Contribution Statement

VP grew the plants; made the experiments, wrote the paper; YBM grew the plants; made the experiments; DMP grew the plants; made the experiments; LR planned the experiments, revised the paper; SE wrote and revised the paper; JMR planned the experiments, wrote and revised the paper.

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Supplemental Table 1 Changes in Fresh Weight (FW) in leaves and roots of *L. sativa* cv. Phillipus and *B. oleracea* cv. Bronco in control plants, and plants exposed to 0.5 mM Zn.

	Leaf FW (g)	Root FW (g)
<i>L. sativa</i> cv. Phillipus		
Control	30.93 ± 4.36	4.31 ± 0.40
0.5 mM Zn	20.65 ± 1.30	1.14 ± 0.12
p-value	*	***
LSD _{0.05}	9.75	0.90
<i>B. oleracea</i> cv. Bronco		
Control	6.48 ± 1.30	1.91 ± 0.14
0.5 mM Zn	5.11 ± 0.55	0.55 ± 0.06
p-value	NS	***
LSD _{0.05}	3.03	0.32

Values are means (n=9) ± standard error. Legend: DW: dry weight; LSD_{0.05}= Fisher's Least Significant Difference (LSD) (p> 0.05); p-values: *, <0,05; **, <0,01; ***, < 0.001. NS: Not Significant.

Chapter 4

This Chapter contains unpublished results.

Effect of Silicon supply in barley plants grown under different Zn nutrition regimes

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Chapter 4 - Effect of Silicon supply in barley plants grown under different Zn nutrition regimes

1 INTRODUCTION

1.1 Zinc in plants

Zinc (Zn), an essential micronutrient, is required for growth and development of plants (Kochian 1993). Zn was identified as a ubiquitous component in both animal and plant tissues, thus promoting the successive investigations. In *Arabidopsis thaliana* 2367 proteins related to Zn have been found (Broadley et al., 2007). Zn is involved in numerous critical processes in all organisms (Dhankar et al 2012, Sinclair and Krämer 2012), since it is able to bind to different cellular ligands such as cysteine (Cys), histidine (His), aspartate (Asp) and glutamate (Glu) (Cakmak et al 2000, Broadley et al 2007). It should be noted that Zn binds to a wide range of other proteins, membrane lipids and DNA / RNA molecules (Broadley et al 2007). In addition, it has been shown that Zn is necessary for the activity of more than 300 enzymes, this being the only metal represented in the six classes of enzymes: hydrolases, isomerases, lyases, ligases, oxidoreductases and transferases (Broadley et al., 2007) (Table 1). Therefore, Zn participates as catalytic and co-catalytic, structural and regulatory ion (Maret 2009) (Table 1 and Fig. 1). Well known examples in plants include enzymes such as carbonic anhydrase, alcohol dehydrogenase, RNA polymerase, carboxypeptidase, alkaline phosphatase and Cu / Zn superoxide dismutase (Guerinot and Eidet 1999, Auld et al 2001, Sriram and Lonchyna 2009) (Table 1). Other investigations showed how Zn can act as a structural ion in Zn fingers, turns and clusters, in transcription factors and in metallothioneins (Cotton et al., 1997).

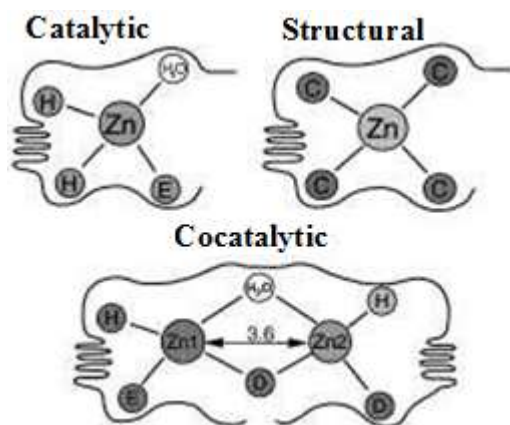


Fig. 1 Zn binding sites in enzymes: catalytic (thermolysin), structural (alcohol dehydrogenase), cocatalytic (*Aeromonas proteolytic* aminopeptidase). Image taken from Auld (2001). The letters D, E and H refer to amino acids, aspartic acid, glutamic acid and histidine, respectively.

	L ₁	X	L ₂	Y	L ₃	Z	L ₄	Solvent
Catalytic zinc								
Oxidoreductases ²								
Alcohol dehydrogenase (horse liver) ³	C ₃₇	20	H ₆₈	106	C ₁₇₄			H ₂ O
Alcohol dehydrogenase (<i>Thermoanaerobium brockii</i>) ⁴	C ₃₇	21	H ₆₉	90	D ₁₅₀		NA ⁵	
Hydrolases								
Carboxypeptidase A (bovine) ^{6,7}	H ₆₈	2	E ₇₂	123	H ₁₉₆			H ₂ O
Thermolysin (<i>Esacillus thermoproteolyticus</i>) ^{8,9}	H ₁₄₂	3	H ₁₄₆	19	E ₁₆₆			H ₂ O
DD carboxypeptidase (<i>Streptomyces albus</i>) ¹⁰	H ₁₉₆	2	H ₁₉₃	40	H ₁₅₂			H ₂ O
Astacin (crayfish) ¹¹	H ₅₂	3	H ₉₆	5	H ₁₀₂			H ₂ O
β -Lactamase (<i>Bacillus cereus</i>) ¹²	H ₆₆	1	H ₈₈	121	H ₂₁₀			H ₂ O
Cytidine deaminase (<i>Escherichia coli</i>) ¹³	C ₁₃₂	2	C ₁₂₉	26	H ₁₀₂			H ₂ O
Alkaline phosphatase (<i>E. coli</i>) ¹⁴	D ₃₂₇	3	H ₃₃₁	80	H ₄₁₂			H ₂ O
Adenosine deaminase (murine) ¹⁵	H ₁₅	1	H ₁₇	196	H ₂₁₄	80	D ₂₉₅	H ₂ O
Lyases								
Carbonic anhydrase II (human) ^{17,16}	H ₉₄	1	H ₉₆	22	H ₁₁₉			H ₂ O
Carbonic anhydrase (spinach) ¹⁸	C ₂₁₃	2	H ₂₁₀	59	C ₁₅₀			H ₂ O
Carbonic anhydrase (<i>Methanosaerina thermophila</i>) ¹⁹	H ₈₁	35	H ₁₁₇	NA ¹⁹	H ₁₂₂			H ₂ O
Novel catalytic zinc sites								
Transferases								
Protein farnesyltransferase (rat) ²⁰	D ₂₉₇	1	C ₂₉₈	62	H ₃₆₂			H ₂ O
Cobalamin-dependent methionine synthase (<i>E. coli</i>) ²¹	C ₂₄₇	62	C ₃₁₀		C ₃₁₁		N/O atom	
Cobalamin-independent methionine synthase (<i>E. coli</i>) ^{21,27}	H ₆₄₁	1	C ₆₄₃	82	C ₇₂₆		N/O atom	
Nonenzymatic								
Ada repair protein (<i>E. coli</i>) ²²	C ₃₈	3	C ₄₂	26	C ₆₉	2	C ₇₂	No
Structural zinc								
Alcohol dehydrogenase (horse liver) ³	C ₉₇	2	C ₁₀₀	2	C ₁₀₃	7	C ₁₁₁	No
Aspartate carbamoyltransferase (<i>E. coli</i>) ²³	C ₁₀₉	4	C ₁₁₄	23	C ₁₃₈	2	C ₁₄₁	No
Zinc finger (Zif268) (mouse) ²⁴	C ₇	5	C ₁₂	12	H ₂₅	4	H ₂₉	No
Glucocorticoid receptor (rat) ²⁵	C ₄₄₀	2	C ₄₄₃	8	C ₄₅₇	2	C ₄₆₀	No
Ferredoxin (<i>Sulfolobus</i> sp.) ²⁶	H ₁₆	2	H ₁₉	14	H ₃₄	41	D ₇₆	No

Table 1 Comparison of the ligands (L₁, L₂, L₃ and L₄) and the spacers (X, Y and Z) between zinc ligands in zinc catalytic and structural sites (Taken from McCall et al., 2000).

In plants, Zn is required at optimal concentrations both for the normal functioning of the cellular metabolism and for the growth of the same (Broadley et al., 2007), since this is involved in numerous physiological processes such as enzyme activation, protein synthesis, carbohydrate metabolism, lipids, nucleic acids and auxins, structural and functional integrity of biomembranes, gene expression and regulation, and reproductive development (pollen formation) (Brown et al., 1993; Marschner 1995). Further Zn performs very critical role in the defense system, composed of metabolites like ascorbic acid (AsA), glutathione and enzymatic scavengers (Asada 1999), and prevents oxidation of vital components (Mukhopadhyay et al. 2013a).

The majority of Zn in the leaf is associated with compounds of low molecular weight, storage metalloproteins, free ions, and insoluble forms associated with the cell wall. In addition, Zn can become inactivated within the cell, either by the formation of ligands or by the formation of complexes with phosphorus. In this sense, Brown et al. (1993) show how, depending on plant species, Zn can be soluble from 58% to 91%.

Zn is absorbed from the soil mainly as free divalent cation (Zn^{2+}), although in the presence of a high pH in the soil, it can be absorbed as a monovalent cation ($ZnOH^+$) (Marschner 1995). In addition, this trace element is usually absorbed by forming complexes with organic ligands (Broadley et al., 2007). However, the absorption of Zn will depend on its bioavailability in the soil. In this sense, there are several factors that affect the absorption of metal, such as: the pH, texture and amount of organic matter in the soil, temperature, light, crop, cultivars and the interaction with different nutrients such as P, N, Fe, Mn, Cu, Co and Cd (Rehman et al., 2012).

First Zn can be strongly adsorbed at the root level or be absorbed. If it is absorbed, it will cross the plasma membrane through active transporters (Rehman et al., 2012), and

it will be transported from the epidermis and cortex to the xylem via simplasto through plasmodesms, being finally released to the apoplast (Broadley et al. 2007). The distribution and transport of Zn can be affected by the adequate supply of this micronutrient, but under normal conditions the transport of Zn does not coincide with the transport of water. Once the aerial part is reached, the Zn can accumulate and later be removed to the young leaves via phloem (Lognecker and Robson 1993). The environmental contamination with heavy metals or metalloids as well as the deficiency of micronutrients (especially Zn) in humans are global problems.

It has been found that Zn deficiency is the most widespread micronutrient deficiency. Zn deficiency in plants occurs in soils with low concentration of available Zn, found in many world regions (Sillanpää 1982). External symptoms occurring in plants with Zn deficit are observed mainly in leaves and usually consist of reduced biomass, internervial chlorosis, necrotic spots, browning, rosette disposal, small and deformed leaves, and growth delay (Alloway 2008). As a result of Zn deficiency several changes in physiological processes occur: reduction in photosynthesis, glycolysis, starch synthesis, protein synthesis activity, membranes destabilization and also flowering and seed production are affected (Brown and Cakmak 1993). Besides these processes, under Zn deficiency nitrogen (N) metabolism is altered and it was found that NO_3^- absorption is reduced and therefore their concentration in the plant (Johnson and Simons 1979).

Zinc is a redox-inactive micronutrient, however, excess of it induces toxicity and impairs growth of plants. Zn is a major industrial pollutant of the terrestrial and aquatic environment (Broadley et al. 2007). Among the main Zn producing regions we can find Asia, followed by Europe and the USA (Fig. 2).



Fig. 2 Principal Zn producers worldwide. The shaded countries of gray color correspond to the main producers ordered from highest to lowest production tons: China, Peru, Australia, India, United States, Canada, Mexico, Kazakhstan, Bolivia and Ireland (Taken from USGS: Mineral Commodity Summary 2011).

Under Zn toxicity conditions, Zn accumulates excessively in plant tissues, causing physiological alterations, inhibiting growth and even producing death (Hoffmann 1983). The toxicity thresholds can be very variable, even within the same species (Broadley et al., 2007). In general, the toxicity symptoms are visible from a concentration higher than 300 mg Zn kg⁻¹ in the dry weight of the leaf, although some crops may show symptoms of toxicity with concentrations less than 100 mg Zn kg⁻¹ of dry weight (Chaney 1993; Marschner 1995).

This effect may be due to the fact that Zn induces, alteration of carbohydrate metabolism (Foy et al., 1978), growth inhibition, decrease in the content of essential nutrients such as Fe (Connolly et al., 2002,2003, Sagardoy et al. 2009), Cu and Mn, oxidative damage to membranes and disturbances of photosynthetic activity in numerous cellular locations such as photosynthetic pigments, electron transport,

Rubisco activity, etc. (Clemens 2001, Broadley et al., 2007; Vassilev et al. 2007). Therefore, plants must regulate the homeostasis of this metal to cope with adverse environmental conditions (Krämer and Clemens 2005), which makes the presence of some tolerance mechanism key for this purpose.

1.2 Hordeum vulgare

Hordeum vulgare (barley) is the world's fourth most important cereal crop. It's a member of the grass family and it is an important model for ecological adaptation.

About two-thirds of the global barley crop is used for animal feed, although for humans it is still the major source of Kcal in several parts of the world.

It's very important as model organism for different reasons:

- Barley is the fourth most important cereal plant in the world
- It has a short life cycle and morphological, physiological, and genetic characteristics
- It is simple to grow in both field and in laboratory
- Barley is diploid ($2n = 2x = 14$) self fertile inbreeding plant with genome size roughly 5.3 billion base pairs divided in 7 chromosomes (Fig. 3)
- Barley genome has been completely sequenced (International Barley Genome Sequencing consortium and also the UK Barley Sequencing Consortium 2012)

Barley genome is one of the largest diploid genomes sequenced to date, and this makes it a natural model for the genetics and genomics analyses for the Triticeae tribe, including wheat and rye. During the last years a great number of studies were done on barley in order to disclose many physiological mechanisms occurring in barley, in order to improve crop yield under biotic and abiotic stress.

1.3 Silicon

Silicon (Si) is the second most abundant element after oxygen in the Earth's crust (Epstein 1999). Although Si has not been considered as an essential element for higher plants, it has been proven to be beneficial for the healthy growth and development of many plant species, particularly graminaceous plants such as rice, sugarcane and cyperaceous plants (Epstein, 1994, 1999; Liang et al., 2005a). Silicic acid, the main Si form used by plants, is the only nutrient element that is not harmful when excessively concentrated in plants (Ma and Yamaji 2006). Silicon concentrations vary greatly in plant aerial parts, ranging from 0.1 to 10.0% of dry weight. This wide variation in Si concentration in plant tissues is attributed mainly to differences in the characteristics of Si uptake and transport (Epstein, 1994). In general, graminaceous plants take up much more Si than other species, while most dicotyledonous plants absorb Si passively and some dicots such as legumes exclude Si from uptake (Liang et al., 2005b). Besides rice, other graminaceous plants such as wheat (Casey et al., 2003), ryegrass (Jarvis, 1987), and barley (Barber and Shone, 1966) and some cyperaceous plants take up Si actively. It has been reported that both active and passive mechanisms are operating in Si uptake and transport in the same Si-accumulator such as rice and maize and intermediate type species such as sunflower and wax gourd with their contribution being dependent upon plant species and external Si concentrations (Liang et al., 2006). In the last decades, several authors demonstrated that Si is beneficial for plants particularly under abiotic (Hattori et al. 2005; Tripathi et al. 2012) and biotic stresses (Epstein 1999; Ma and Yamaji 2006). In most studies concerning silicon supplementation, Si is supplied as calcium silicate or as sodium metasilicate at concentrations lower or equal to 5 mM (Eraslan et al., 2008; Miao et al., 2010).

Literature data indicate that Si concentration equals 2 mM corresponds to the limit of silica solubility in water (Korndörfer and Lepsch, 2001).

Si strengthens cell walls augmenting suberization, lignification and silicification in order to improve the mechanical support for monocots and pteridophytes by (He et al. 2013). Particularly, the deposition of silicic acid within the apoplast develops an amorphous silica barrier that represents a defence against biotic and abiotic stresses (Guerriero et al. 2016).

2 OBJECTIVES

Zn is an essential micronutrient necessary for plants, animals and microorganisms. However, due to the increase in industrial activities, the depositions of this trace element have increased. Under these circumstances, Zn can accumulate excessively in plant tissues causing physiological alterations and inhibition of growth. On the other hand, Zn deficiency in soils represents up to 30% of the cultivated land. Under this situation can cause an acute deficiency in higher plants which can lead to an increase in Zn malnutrition in humans reaching affect the health of more than three billion people around the world. Phytoremediation of metal/metalloids contaminated soils such as biofortification of mineral micronutrients in food crops for the benefit of human nutrition represent two potential biotechnological applications.

Thus, the understanding in the absorption of minerals, distribution, metabolism and tolerance, and the molecular mechanisms responsible for the processes, are crucial for both applications. Therefore the fundamental objectives of this Doctoral Thesis have been the following:

1. Realization of a study of the different strategies in plants of *Hordeum vulgare* grown under deficiency (0.01 μM) and toxicity of Zn (100 μM), such as the metabolism of carboxylates and oxidative and glutathione metabolism as well as osmoprotective compounds in order to define key physiological processes to improve the stress condition of plants.
2. Evaluate the effects of 1 mM Si dose on plants of *Hordeum vulgare* grown under deficiency (0.01 μM) and toxicity of Zn (100 μM), in order to ascertain if this silicate could be used in programe of biofortification and phytoremediation.

Hydroponic system was used to precisely control the Si dosage and mitigate the external contaminations.

3 MATERIALS AND METHOD

3.1 Plant material and growth conditions

Seeds of barley (*Hordeum vulgare*, Nure) were germinated on moistened paper for approximately 7 days. The seedlings were grown in a growth chamber under the following controlled environmental conditions: Relative humidity 60–80%; Day/night temperatures 28/19 °C; 16/8 h photoperiod at a photosynthetic photon flux density (PPFD) of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ (measured at the top of the seedlings with a 190 SB quantum sensor, LI-COR Inc., Lincoln, Nebraska, USA). Under these conditions, after 7 days of germination, the plants were grown in hydroponic cultivation with a nutrient solution at pH 6.5 (Rigano et al. 1996) (Table 2).

KH ₂ PO ₄	10 mM
K ₂ HPO ₄	10 mM
K ₂ SO ₄	1.25 mM
MgCl ₂	1 mM
Oligo-elements	10 mL/L
FeSO ₄ EDTA	3.5 mg/L
CaCl ₂	1 mM
(NH ₄) ₂ SO ₄	2.5 mM
H ₂ O	Final volume 1L
Oligoelements	
KCl	190 mg/L
H ₃ BO ₃	80 mg/L
CuSO ₄ x 5 H ₂ O	6 mg/L
MnSO ₄	43 mg/L
(NH ₄) ₆ Mo ₇ O ₂₄ x 4H ₂ O	1 mg/L

Table 2 Composition of *Hordeum vulgare* nutrient hydroponic solution.

3.2 Experimental design

Treatments started after the 7 days of germination and were maintained for 14 days. The control treatment received the growth solution, described in Table 2, containing a 1 μM ZnSO_4 concentration; this solution was renewed every two days. Zn deficiency treatment received the growth solution containing oligo-elements deprived of ZnSO_4 plus 0.01 μM ZnSO_4 , this solution was renewed every two days.

The Zn toxicity treatment received the growth solution containing oligo-elements deprived of ZnSO_4 plus 100 μM ZnSO_4 , this solution was renewed every two days.

Silicon as Calcium Silicate was added at a 1 mM concentration in nutrient solutions at the beginning of hydroponic growth (Fig. 3).

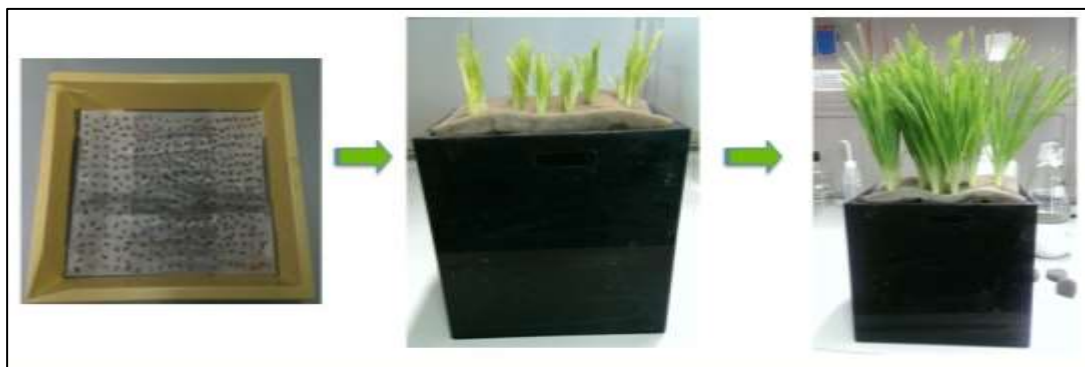


Fig. 3: Steps of hydroponic growth

3.3 Plant sampling and determination of the relative growth rate (RGR)

Plants of each treatment (14 days after germination) were divided into roots and leaves, washed with distilled water, dried on filter paper and weighed, thereby obtaining fresh weight (FW). Half of leaves from each treatment were frozen at $-30\text{ }^{\circ}\text{C}$ for further work and biochemical assays and the other half of the plant material was lyophilized for 48 h to evaluate the dry weight (DW) and the subsequent analysis of the concentrations of nutrients. To determine the relative leaf growth rate (RGR), leaves from three plants per cultivar were sampled on day 1 after germination, immediately before starting the stress

treatment (Ti). The leaves were dried in a forced-air oven at 70 °C for 24 h, and the dry weight (DW) was recorded as grams per plant. The remaining plants were sampled 14 days after germination (14 days of treatments, Tf). The relative growth rate was calculated from the increase in leaf DW at the beginning and at the end of the treatment, using the equation:

$$\text{Relative Grow Rate (RGR)} = (\ln DW_{\text{final}} - \ln DW_{\text{initial}}) / (\text{Time}_{\text{final}} - \text{Time}_{\text{initial}})$$

3.4 Zn concentration

Zinc was determined using a sample of 150 mg dry material subjected to mineralization by wet digestion according Wolf (1982). To carry out this assay, dry leaves were ground and mineralized with a mixture of nitric acid (HNO₃)/perchloric acid (HClO₄) (v/v) and H₂O₂ at 30%. From the resulting mineralization, and after the addition of 20 ml of milli-Q H₂O, elements concentrations were determined by ICP-MS (X-Series II; Termo Fisher Scientific Inc., Waltham, MA, USA). Internal standards included Sc (50 ng ml⁻¹) and Ir (5 ng ml⁻¹) in 2% TAG HNO₃. External multi-element calibration standards (Claritas-PPT grade CLMS-2, SPEX Certi-Prep Ltd, Stanmore, Middlesex, UK) included Al, As, Ba, Bi, Cd, Co, Cr, Cs, Cu, Fe, Mn, Mo, Ni, Pb, Rb, Se, Sr, U, V, and Zn, in the range 0–100 µg l⁻¹, and Ca, Mg, K, and Na in the range 0–100 mg l⁻¹.

3.5 N efficiency parameters (NUE)

NUE parameters were calculated as follow:

N uptake efficiency (NUpE) was calculated as total N accumulation divided by root DW (mg Ca g⁻¹ RDW).

N utilisation efficiency (NUtE) was calculated as leaf tissue DW divided by N concentration (g² LDW mg⁻¹ Ca).

3.6 Extraction and determination of inorganic nitrogen and nitrogen compounds

Total nitrogen concentration (TNC) was calculated as the sum of NO_3^- and total reduced N. NO_3^- was analysed from an aqueous extraction of 0.1 g of DW in 10 ml of Millipore-filtered water. A 100 μl aliquot was taken and added to 10% (w/v) salicylic acid in sulfuric acid at 96%, measuring the NO_3^- concentration by spectrophotometry as performed by Cataldo et al.(1975) .

For total reduced N determination, a sample of 0.1 g DW was digested with sulfuric acid and H_2O_2 . After dilution with deionized water, a 1-ml aliquot of the digest was added to the reaction medium containing buffer 1 (sodium salicate/sodium nitroprusside) and buffer 2 (sodium hydroxide and sodium dichloroisocyanurate). Samples were incubated at 37°C for 15 min, and total reduced N was measured by spectrophotometry using spectrophotometer (Infinite 200 Nanoquant, Tecan, Switzerland) according Krom (1980).

To determinate the concentration of soluble NH_4^+ , the same aqueous extraction of NO_3^- determination was done, but the analysis was carried out following the method of Krom (1980).

3.7 N metabolism enzymes extractions and assays

Leaves were ground in a mortar at 0°C in 50 mM KH_2PO_4 buffer (pH 7.5) containing 2 mM EDTA, 2 mM dithiothreitol (DTT), and 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP). The homogenate was centrifuged at 30,000 $\times g$ for 20 min at 4°C. The resulting extract (cytosol and organelle fractions) was used to measure enzyme activity of NR, GOGAT, and GDH.

3.7.1 Nitrate reductase activity

Nitrate reductase (NR) assay followed the methodology of Kaiser and Lewis (1984). The obtained supernatant was added to the reaction mixture containing: 100 mM KNO₃, 2 mM NADH, 10 mM cysteine and 10 mM MgCl₂ in a solution 100 mM KH₂PO₄ (pH 7,5) and the mixture was incubated at 30°C during 30 min. After it was used 1 mM zinc acetate as a stop reagent. According to the method of (Hageman and Hucklesby, 1971), the detection of NO₂⁻ produced was colorimetrically determined at 540 nm after azo-coupling with sulfanilamide at 1% in 1,5 M HCl and naphthylthylenediamine dihydrochloride (NNEDA) at 0,02% (p/v) in 0,2 M HCl.

3.7.2 Glutamate synthetase activity

Glutamate synthetase (GOGAT) activity was assayed spectrophotometrically at 30°C by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986), always within 2 h of extraction. The supernatant obtained with the previous extraction was added to the reaction mixture 15 mM aminooxyacetate, 1,5 mM NADH, 18,75 mM α- ketoglutarate and 75 mM L- glutamine in a buffer 100 mM KH₂PO₄ (pH 7,5). The decrease in absorbance was recorded for 5 min.

3.7.3 Glutamate dehydrogenase activity

Glutamate dehydrogenase (GDH) activity was assayed by monitoring the oxidation of NADH at 340 nm, essentially as indicated by Groat and Vance (1981) and Singh and Srivastava (1986). The reaction mixture consisted of 50 mM KH₂PO₄ buffer (pH 7.5) with 200 mM NH₄ sulfate, 0.15 mM NADH, 2.5 mM 2-oxoglutarate, and enzyme extract. The decrease in absorbance was recorded for 6 min.

3.7.4 Glutamine synthetase activity

Glutamate synthetase (GS) was determined by an adaptation of the hydroxamate synthetase assay published by Kaiser and Lewis (1984). Leaves were ground in a mortar at 0°C in 50 ml maleic acid-KOH buffer (pH 6.8) containing 100 mM sucrose, 2% (v/v) β -mercaptoethanol, and 20% (v/v) ethylene glycol. The homogenate was centrifuged at $30,000 \times g$ for 20 min AT 4°C. The resulting extract was used to measure enzyme activity of GS. The reaction mixture used in the GS assay was composed of 520 mM imidazole-HCl buffer (pH 7.8) with 4 mM EDTA, 150 mM L-sodium glutamate, 45 mM $MgSO_4 \cdot 7H_2O$, 30 mM hydroxylamine, 10 mM ATP, and enzyme extract. After incubation at 28°C for 30 min, the formation of glutamylhydroxamate was colorimetrically determined at 540 nm after complexing with acidified ferric chloride (Wallsgrave *et al.* 1979).

3.8 Aspartate aminotransferase activity

Aspartate aminotransferase (AAT) activity was assayed spectrophotometrically at 340 nm using the method published by (Gonzalez *et al.* 1995). AAT enzyme was extracted in identical conditions to GS. The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 8), 4 mM $MgCl_2$, 10 mM aspartic acid, and enzyme extract. The decrease in absorbance was recorded for 3 min.

3.9 Determination of photorespiration enzymes activity

3.9.1 Glyoxylate oxidase activity

For GO determination, fresh leaf tissue (0.1 g) was ground in a chilled mortar with PVPP and 1 ml of 50 mM Tris-HCl buffer (pH 7.8) with 0.01% Triton X-100 and 5 mM DTT. The homogenate was centrifuged at $30,000 \times g$ for 20 min. The supernatant was decanted and immediately used for the enzyme assay. GO was assayed as described by Feierabend and Beevers (1972) with modifications. A volume of assay mixture containing 50 mM Tris-HCl buffer (pH 7.8), 0.009% Triton X-100, 3.3 mM phenylhydrazine HCl (pH 6.8), 50 μ l plant extract, and 8 mM glycolic acid (neutralized to pH 7 with KOH) was used to start the reaction. GO activity was determined by following the formation of glyoxylate phenylhydrazone at 324 nm for 3 min.

3.9.2 Glutamate glyoxylate aminotransferase activity

For determination of GGAT activity, leaves were ground in a chilled mortar in 100 mM Tris-HCl buffer (pH 7.3) containing 0.1% (v/v) Triton X-100 and 10 mM DTT. The homogenate was centrifuged at $20,000 \times g$ for 10 min AT 4°C. The resulting extract was used to measure enzyme activity. GGAT activity was measured by coupling the reduction of 2-oxoglutarate by NADH in a reaction catalyzed by GDH. The extraction medium was optimized for the enzyme activities such that they could be extracted together using the same method (Hoder *et al.* 1983). The reaction was assayed in a mixture containing 100 mM Tris-HCl (pH 7.3), 20 mM glutamate, 1 mM glyoxylate, 0.18 mM NADH, 0.11 mM pyridoxal-5-phosphate, 83 mM NH₄Cl, and 0.3 U GDH in a final volume of 0.6 ml (Igarashi *et al.* 2006).

3.10 Determination of N derived compounds

3.10.1 Proline content

To determine the Pro concentration, 0.1 g of the sheet was homogenized in 5 ml of 96% ethanol. The insoluble fraction was washed with 5 ml of ethanol at 70%. The extract was centrifuged at $3500 \times g$ for 10 min and the supernatant was preserved at 4°C for the determination of Pro according to Irigoyen et al. (1992). A 1 ml aliquot of the supernatant was taken, ninhydrin reagent (ninhydrin, 6 M phosphoric acid, 60% glacial acetic acid) and 99% glacial acetic acid (2.5 ml) was added and placed in a bath of water at 100°C. After 45 min, the tubes were cooled on ice and 5 ml of benzene was added. After 10 minutes, the absorbance of the organic phase was measured at 515 nm.

3.10.2 Glycine betaine content

The GB concentration was determined following the method described by Grieve and Grattan (1983). The GB was extracted from 38 mg of lyophilized plant material and dissolved in 1.5 ml of water to which it was subjected to an aqueous extraction. The extract was filtered and 300 μ l of 2N H₂SO₄ was added, the solution was incubated at 4 ° C for 16 h and then centrifuged at $9000 \times g$ for 15 min at 0 ° C. The obtained pellet was resuspended in 1,2-dichloroethane. After 2 h the GB concentration was determined by measuring the absorbance at 365 nm by spectrophotometry.

3.11 Soluble AAs analysis

Soluble AAs were extracted following the method of Bielecki and Turner (1966) with some modifications. 0.1 g of fresh leaves were homogenised in 1 ml of MCW (methanol: chloroform: water, 12:5:1). 50 µl of L-2 aminobutyric acid was added as an internal standard. The mixture was centrifuged at 5,000 rpm for 10 min. To the resulting supernatant were added 700 µl of Milli-Q water and 1.2 ml of chloroform and incubated 24 h at 4 °C. Then, the aqueous phase was obtained, which was lyophilized and the resulting extract was diluted with 0.1 M HCl. Instrumental analysis of soluble AAs was carried out using the precolumn AccQ Tag Ultra Derivatization Kit (Waters, Milford, MA, USA). Derivatization was performed according to the manufacturer's protocol. For derivatization, 60 µl of borate buffer was added to 10 µL of the sample, 10 µL 0.1 N NaOH and 20 µL reconstituted AccQ•Tag Ultra Reagent. LC fluorescence analysis was performed on the Waters Acquity® UPLC System equipped with the Acquity fluorescence detector. UPLC separation was performed on the AccQ Tag Ultra column (2.1 x 100 mm, 1.7 µm) from Waters. The flow rate was 0.7 mL min⁻¹, and the column temperature was kept at 55°C. The injection volume was 1 µL, and the detection was set at a 266-nm excitation wavelength and a 473-nm emission wavelength. The solvent system consisted of two eluents: 1:20 Dilution of AccQ Tag Ultra eluent A concentrate and AccQ Tag Ultra eluent B. The profile was as follows: 0–0.54 min, 99.9% A and 0.1% B; 5.74 min, 90.9% A and 9.1% B; 7.74 min, 78.8% A and 21.2% B; 8.04 min, 40.4% A and 59.6% B; 8.05–8.64 min, 10% A and 90% B; 8.73–9.50 min, 99.9% A and 0.1% B.

3.12 Determination of Photosynthetic parameters

3.12.1 Leaf pigment concentrations

The concentration of photosynthetic pigments was analyzed by the method of Wellburn et al. (1994) with certain modifications. 0.1 g of frozen plant material was macerated in 1 ml of methanol. Subsequently, the extract was centrifuged at 5000 x g for 5 min. The absorbance was measured at 3 different wavelengths: 666 nm, 653 nm and 470 nm. The chlorophyll a (Chl a), chlorophyll b (Chl b), and carotenoids were estimated by using the equation of Lichtenthaler [19]:

$$\text{Chlorophyll a (Chl a)} = 15.65 \times A_{666\text{nm}} - 7.34 \times A_{653\text{nm}}$$

$$\text{Chlorophyll b (Chl b)} = 27.05 \times A_{653\text{nm}} - 11.21 \times A_{666\text{nm}}$$

$$\text{Carotenoids} = (1000 \times A_{470\text{nm}} - 2.86 \times \text{Chl a} - 129.2 \times \text{Chl b}) / 221$$

Total Chl was calculated as the sum of Chl a and Chl b.

3.12.2 Chlorophyll by SPAD value

The chlorophyll content in the leaves was measured using the chlorophyll meter SPAD-502 (Konica Minolta Sensing Inc, Japan). Three measurements were made per leaf, nine leaves were analyzed for each treatment and the average of the measurements was calculated.

3.12.3 Chl a fluorescence analysis

Plants were adapted to dark for 30 min before measurements using a leaf clip holder that was allocated in each fully expanded leaf. Chl a fluorescence kinetics was determined using the Handy PEA Chlorophyll Fluorimeter (Hansatech Ltd., King's Lynn, Norfolk, UK); the OJIP transients were induced by red light (650 nm) with 3000

$\mu\text{mol photons m}^{-2}\text{s}^{-1}$ light intensity and recorded by the instrument. OJIP transients data were analysed using the JIP-test (Strasser et al, 2000).

Measurements were conducted with six plants of fully expanded leaves at midstem position. Parameters employed to study the energy flow and photosynthetic activities by JIP-test were: initial fluorescence (F_o), maximum fluorescence (F_m), variable fluorescence ($F_v = F_m - F_o$), area above the fluorescence curve between F_v and F_m (Area), fluorescence value at 300 μs (K step), maximum quantum yield for primary photochemistry ($\Phi_{P_o} = F_v/F_m$), performance index (PI_{ABS}), proportion of active reaction centres (RCs) (RC/ABS), efficiency/probability with which a PSII trapped electron is transferred from Q_A to Q_B (Ψ_o), maximum quantum yield of electron transport ($\Phi_{E_o} = \text{ETo}/\text{ABS}$), fluorescence value at 300 μs (K step) and dissipated energy (DI) by excited RC (DI / RC) (Strasser et al., 1999, Strasser et al., 2000).

3.13 Concentration of oxidative stress indicators (MDA, H_2O_2 and O_2^-)

H_2O_2 concentration was colorimetrically determined according to Mukherjee & Choudhuri (1983). Leaf samples were extracted with cold acetone. An aliquot of 1 mL of the extract was mixed with 200 μL of 0.1% titanium dioxide in 20% H_2SO_4 (v: v) and the mixture was centrifuged at 6000 g during 15 min. The intensity of yellow colour of the supernatant was measured at 415 nm. The concentration of H_2O_2 was calculated from a standard curve of H_2O_2 . The result of H_2O_2 concentration was expressed as $\mu\text{g g}^{-1}$ DW.

The concentration of O_2^- in the leaves was measured colorimetrically according to Barrameda-Medina et al. (2014). 0.1 g of plant material was ground and 300 μL of 50 mM phosphate buffer was added. The mixture was centrifuged at 10000 g for 15 minutes. From the supernatant, 250 μL were taken and 50 mM phosphate buffer and

250 μL of 10 mM hydroxylamine were added to the extract. The mixture was incubated for 20 min at 25 ° C. Subsequently, 60 μL was taken from the supernatant and 180 μL of 17 mM sulfonic acid and 180 μL of 7 mM α -1-naphthylamine were added, and the mixture was incubated 1 h at room temperature. At the end of the incubation time, the intensity of the color was measured at 530 nm. The concentration of O_2^- was calculated from a standard curve of O_2^- .

3.14 Activity of antioxidant enzymes (APX, CAT and GR)

The CAT activity was determined as described by Badiani et al. (1990). 0.1 g of plant material was ground in 1 mL of 25 mM sodium phosphate buffer and centrifugated at 11500 x rpm for 20 min at 4°C. The extract was added to a reaction solution composed by 25 mM buffer HEPES-HCl (pH 7.8), 0.8 mM EDTA-Na and 40 mM H_2O_2 . CAT activity was determined by analyzing H_2O_2 consumption (molar extinction coefficient 39.4 $\text{mM}^{-1}\text{cm}^{-1}$) at a wavelength of 240 nm for 3 min.

The APX and GR enzymes assay was performed according to Rao et al. (1996). APX activity was determined by recording the change in absorbance at 290 nm for 3 minutes of a reaction mixture containing 100 mM potassium phosphate buffer (pH 7.5), 0.5 mM AsA, 0.2 mM H_2O_2 and 0.75 mL enzyme extract. On the other hand, the GR activity was measured following the oxidation of NADPH at 340 nm for 3 minutes in a reaction mixture containing 100 mM Tris-HCl (pH 7.8), 2 mM $\text{Na}_2\text{-EDTA}$, 0.2 mM NADPH, 0.5 mM of GSSG and 0.75 mL of enzymatic extract.

3.15 Concentration of antioxidant non-enzymatic systems (AsA and GSH)

For the extraction and quantification of reduced AsA, the method of Law et al. (1992) was used. This method is based on the reduction of Fe^{3+} to Fe^{2+} by the AsA in acid solution. 0.5 g of frozen plant material was homogenized in 5 mL of 5% metaphosphoric acid (w / v) and subsequently centrifuged at 4 ° C for 15 min. Then 0.2 mL of supernatant was added to a test tube together with 0.5 mL of 150 mM sodium phosphate buffer (pH 7.5) and 0.1 mL of 10 mM distilled H_2O . The mixture was shaken and incubated at room temperature and in the dark for 10 minutes. Then 0.1 mL of N-ethylmaleimide 0.5% (w / v), 0.4 mL of orthophosphoric acid 44% (v / v), 0.4 mL of 2,2'-bipyridyl 4% (p / v) were added. v) in 70% ethanol and 0.2 mL of 3% FeCl_3 (w / v). The test tubes were then shaken and incubated at 40 ° C and in the dark for 40 minutes. Finally, the absorbance at 525 nm was measured against a standard curve of AsA in which the same procedure was followed.

For the reduced GSH concentration, 0.2 g of leaf were macerated in 1 mL of 0.2 M HCl and centrifuged at 16,000 g for 10 min. An aliquot of 500 μL was collected and 500 μL of sodium phosphate buffer (pH 7.5) was added to it. From this mixture, an aliquot of 25 μL was extracted and were added 90 μL of sodium phosphate buffer, 10 μL of 10 mM EDTA, 10 μL of 10 mM NADPH, 10 μL of 6 mM DTNB, 35 μL of distilled H_2O and 10 μL of GR 10 UD / mL. The reduced GSH concentration was measured at 412nm. (Gronwald et al., 1987).

3.16 Extraction and assays of carboxylate metabolism enzymes

Extracts for measuring enzyme activities were made following the method of Li (2000), modified by grinding 0.1 g of leaves in 1 ml of extraction buffer containing 1 mM EDTA-Na, 10% glycerol, 1% TritonX-100, 5 mM DTT and 1% polyvinylpyrrolidone (PVP) in 100 mM Tris-HCl pH 8.0. The slurry was centrifuged for 5 min at 14700 rpm and 4 °C, and the supernatant was collected and analysed immediately. The activities of all enzymes were analysed in 0.2 ml (final volume) of the media indicated below. CS (EC4.1.3.7) activity was assayed spectrophotometrically by monitoring the reduction of acetyl coenzyme A (CoA) to Co A with 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) at 412 nm (Srere, 1969). The reaction was carried out in 0.1 mM DTNB, 0.36 mM acetyl CoA, 0.5mM oxalate and 100 mM Tris-HCl, pH8.1. PEPC (EC4.1.1.31) activity was measured in a coupled enzyme assay with the MDH in 2 mM phosphoenolpyruvate (PEP), 10 mM NaHCO₃, 5 mM MgCl₂, 0.16 mM NADH and 100 mM of N,N-bis[2-hydroxyethyl]glycine (Bicine)-HCl, pH 8.5 (López-Millán et al., 2001). FUM (EC4.2.1.2) was assayed following the increase in optical density at 240 nm due to the formation of fumarate in 50 mM malate and 100 mM phosphate buffer, pH 7.4 (Bergmeyer et al., 1974). Finally MDH (EC 1.1.1.37) activity was determined with oxalate as substrate by measuring the decrease in absorbance at 340 nm due to the enzymatic oxidation of NADH (Dannel et al., 1995). The reaction was carried out with 0.1 mM NADH, 0.4 mM oxalate and 46.5 mM Tris-HCl, pH 9.5. The protein concentration of the extracts was determined using bovine-serum albumin as the standard (Bradford, 1976).

3.17 Concentrations of organic anions

Approximately 0.1 g of samples frozen leaves were homogenized with 1 ml milliQ water. The resulting mixture was centrifuged at 2.000 rpm for 25 min and diluted 1:5 with 10 mM KH_2PO_4 pH 2.6, then filtered through a $0.45\mu\text{m}$ membrane filter and the samples were analyzed by HPLC–DAD. The analysis were carried out using an Agilent HPLC 1100 series equipped with a diode array detector and mass detector in series (Agilent Technologies, Waldbronn, Germany). Samples were injected into a Luna C18 column ($150\text{ mm} \times 1.0\text{ mm}$, $3\ \mu\text{m}$ particle size; Phenomenex, Macclesfield, UK) operating at 25°C . Mobilephase (10 mM KH_2PO_4 , pH 2.6) was pumped with a 0.6 ml min^{-1} flow rate. Organic anions were detected at 210 nm. Peaks corresponding to oxalate, citrate, and malate, were identified by comparison of their retention times with those of known standards from Bio-Rad and Sigma. Quantification was made with known amounts of each organic anions using peak areas (Scherer et al., 2012).

3.18 Proteolytic activity

For the proteolytic activity, 0.1 g of leaf was macerated in 1 mL of 50 mM Tris-HCl buffer (pH 7.5) and centrifuged at $16.000 \times g$ for 15 min. An aliquot of 200 μL was collected and 250 μL of azocasein dissolved in 0.1 M Tris-HCl buffer (pH 8.3) was added to it. The mixture was incubated at 37°C for 3 hours. After this time, 1 mL of 10% trichloroacetic acid (TCA) was added to stop the reaction. The extract was incubated in ice for 10 min and centrifugated at $3000 \times g$ for 20 min. An aliquot of 750 μL of the supernatant was collected in a test tube and was added to 750 μL of a buffer NaOH. The tube was incubated for 30 min and finally, proteolytic activity was determined by analyzing azocasein consumption (molar extinction coefficient $1.652\text{ M}^{-1}\text{cm}^{-1}$) at a wavelength of 440 nm.

3.19 Proteins determination

The concentration of proteins in the enzymatic extracts was determined by the method of Bradford (1976) using bovine albumin serum as standard.

The Bradford protein assay is a spectroscopic procedure and a colorimetric protein assay based on the absorbance shift of the dye Coomassie Brilliant Blue G-250 at 595nm. The increase of absorbance at 595 nm is proportional to the amount of bound dye to aromatic aminoacid residues present in the protein chain, and thus to the amount of protein in the sample, referred to a standard curve obtained using bovine serum albumin (BSA) as standard protein (Fig. 4)

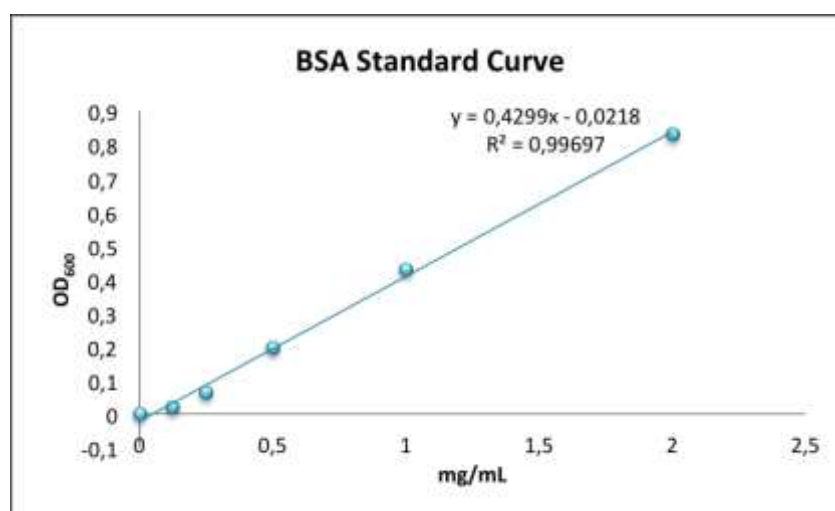


Fig. 4 BSA standard curve for proteins determination.

3.20 Statistical analysis

All analyzes were repeated in triplicate and the results were statistically evaluated using a simple and multifactorial ANOVA variance analysis with a 95% confidence interval. The differences between the means of the treatments were compared using the Fisher's least significant differences (LSD) test at a 95% probability level. Significance levels were expressed as: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; NS not significant.

4 RESULTS

4.1 Biomass and relative growth rate

Plants grown under Zn deficiency (0.01 μM ZnSO_4) or toxicity (100 μM ZnSO_4) presented a relevant decrease – about 40% - of leaf biomass with respect to control plants, from 41 to 24-26 mg dry weight /plants. The application of Si under both conditions of Zn nutrition significantly increased foliar biomass to 32 mg (+23%). Differently, when Si was added to the growth medium containing control doses of Zn, the silicate negatively affected aerial biomass, which remained about 30 mg, about 26% less than control plants without Si added.

Foliar relative growth rate (RGR) followed the same behaviour observed for biomass.

Limited or excessive Zn nutrition induced an appreciable decrease (-20% and -30%) of RGR with respect to optimal conditions. Si supply decreased the relative growth rate from 0.104 mg/day to 0.082 mg/day (-20%) in leaves treated with control Zn levels. Contrarily, in Zn stressed plant, Si nutrition determined a slight but significant increment, respectively of 13% (deficiency) and 28% (toxicity) to a value of 0.090 mg/day (Table 3).

	LEAF BIOMASS (mg DW)			LEAF RELATIVE GROWTH RATE (mg/day)		
	0.01 μ M Zn	1 μ M Zn	100 μ M Zn	0.01 μ M Zn	1 μ M Zn	100 μ M Zn
0 mM Si	24.1 \pm 0.97 ^b	41.1 \pm 0.86 ^a	26.6 \pm 0.63 ^b	0.082 \pm 0,004 ^b	0.104 \pm 0001 ^a	0.071 \pm 0.001 ^b
1 mM Si	32.1 \pm 0.93 ^a	30.6 \pm 0.91 ^b	31.7 \pm 2.02 ^a	0.093 \pm 0.003 ^a	0.082 \pm 0.001 ^b	0.091 \pm 0.001 ^a
P-valor	***	***	**	*	***	***
0.01 μM Zn		28.1 ^b			0.088 ^b	
1 μM Zn		35.8 ^a			0.093 ^a	
100 μM Zn		29.2 ^b			0.081 ^c	
P-value		***			***	
LSD_{0.05}		1.88			0.001	

Table 3 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the parameters foliar biomass (mg DW) and foliar relative growth rate (mg/day) in barley leaves. Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**), and p/<0.001 (***).

4.2 Zinc concentration

Zn deficiency caused a relevant diminution of foliar concentration of zinc (-40%). On the other hand, when toxic levels of Zn were added to the growth medium, plants accumulated zinc, thus resulting in an increased level of foliar Zn (from 22.5 $\mu\text{g g}^{-1}\text{DW}$ to 123 $\mu\text{g g}^{-1}\text{DW}$ – about 5-folds). Si supply to the plant did not affect the controls but significantly increased Zn uptake in plants grown under Zn deficiency, thus maintaining the Zn at the same level as in control plants. Si application in Zn toxic leaves slightly diminished the levels of Zn (- 10%). (Table 4)

ZINC CONCENTRATION ($\mu\text{g g}^{-1}\text{DW}$)			
	0,01 $\mu\text{M Zn}$	1 $\mu\text{M Zn}$	100 $\mu\text{M Zn}$
0 mM Si	13.50 \pm 0.87 ^b	22.51 \pm 2.61 ^a	123.1 \pm 1.01 ^a
1 mM Si	24.00 \pm 0.22 ^a	18.00 \pm 1.73 ^a	109.5 \pm 2.61 ^b
P-value	***	NS	**
0,01 $\mu\text{M Zn}$	18.75 ^b		
1 $\mu\text{M Zn}$	20.25 ^b		
100 $\mu\text{M Zn}$	116.25 ^a		
P-value	***		
LSD _{0.05}	3.02		

Table 4 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the parameter Zn concentration ($\mu\text{g g}^{-1}\text{DW}$) in barley leaves. Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by $p > 0.05$: NS (not significant), $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

4.3 Nitrogen uptake efficiency and nitrogen utilization efficiency

Barley plants subjected to both deficiency and toxicity conditions decreased their NUpE values in comparison to control plants; however, this decrease was more remarkable in Zn deficient plants (-76%) respect to Zn toxic plants (-64%). In contrast, Si application enhanced NUpE value under both Zn deficiency and toxicity, inducing a respective increase of 86% and 48%, levels lower, but near to control plants. Interestingly, silicon decreased NUpE under Zn control conditions the value from 47.88 mgN/gDW_{root} to 28.80 mgN₂/gDW_{root}.

Both Zn deficiency and toxicity increased their NUtE values with respect to control plants, raising the value from 1.7 mgDW/mgN to 2.08 mgDW/mgN₂.

NUtE value was higher in plants grown with Si and control Zn dose than in plants without Si, showing an increase of 30%. However Si did not produced significant effects in NUtE when plants received deficient or toxic Zn doses (Fig. 5).

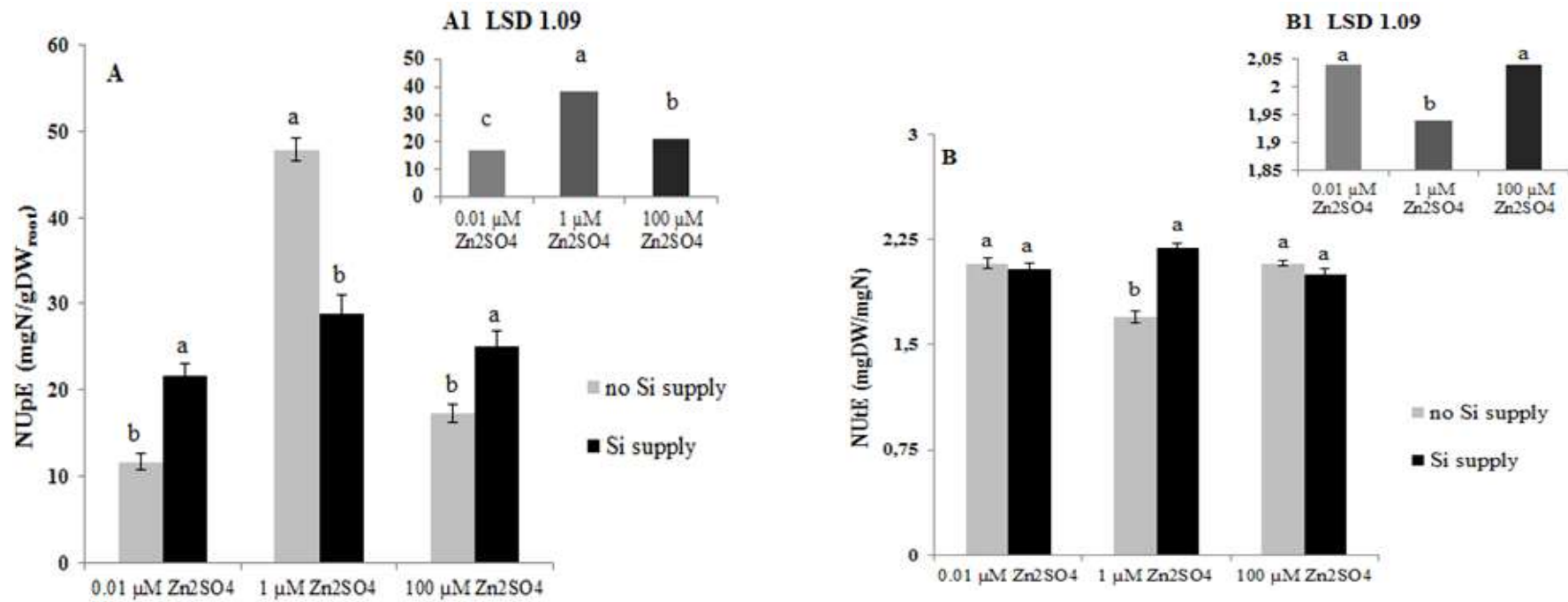


Fig. 5 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the parameters NUPE (mgN/gDWraiz) (A), and NUTE (mgDW/mgN) (B) in barley leaves. The columns values are mean \pm standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05). A1 and B1 Values are means (control +Si supply n = 18) and differences between means were compared using LSD test (P =0.05). Levels of significance are represented by NS (non-significant) P > 0.05; * P < 0.05; ** P < 0.01 and *** P < 0.001 relative to the control.

4.4 N metabolism and Photorespiration

4.4.1 Effects of Zn on inorganic and organic nitrogen

Barley plants subjected to Zn deficiency and toxicity showed an increase – about 30% - in free NO_3^- respect to control. Silicon supply increased free intracellular nitrate in comparison to plants without Si, both in Zn control and Zn deficient nutrition. Similarly, but with a lower entity (25%), Si supply induced a further increase of nitrate content in Zn toxicity treatment.

Free NH_4^+ levels were higher (+30%) in plants grown under Zn deficiency with respect to controls. Toxic Zn supply raised, in a lower but equally significant rate, the levels of free ammonium compared to controls. Si supply affected differently depending on the Zn dose considered. Thus, Si decreased about 15% NH_4^+ in Zn deficient plants, whereas increased it in control and Zn toxicity plants with a variation of 5-13%. (Table 5)

Organic N concentration decreased in barley plants when low and high Zn doses were applied to plants with respect to controls by 66% and 52%, respectively. In contrast, Si application increased 30-35% organic nitrogen under both low and high Zn doses. Silicon induced a stabilisation of the organic nitrogen in all conditions tested to levels of 10-15 $\text{mg g}^{-1}\text{DW}$.

Total nitrogen content (TNC) was higher in control plants, decreased in plants under Zn toxicity, and was lower under Zn deficiency. Si application produced similar effects as for organic N, increasing TNC levels in both plants treated with Zn deficiency (+50%) and toxicity (+30%), whereas reducing in control plants (-38%) (Table 6).

	NO₃⁻ (mg g⁻¹DW)			NH₄⁺ (mg g⁻¹DW)		
	0.01 μM Zn	1 μM Zn	100 μM Zn	0.01 μM Zn	1 μM Zn	100μM Zn
0 mM Si	2.42±0.12 ^b	1.89±0.14 ^b	2.37±0.18 ^b	1.96±0.04 ^a	1.44±0.03 ^b	1.68±0.04 ^b
1 mM Si	3.86 ±0.22 ^a	3.03±0.08 ^a	2.96±0.09 ^a	1.68±0.01 ^b	1.52±0.01 ^a	1.91±0.03 ^a
P-value	***	***	*	*	***	***
0.01 μM Zn		3.14 ^a			1.82 ^a	
1 μM Zn		2.46 ^b			1.49 ^b	
100 μM Zn		2.67 ^b			1.79 ^a	
P-value		***			***	
LSD_{0.05}		0.25			0.05	

Table 5 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the concentration of nitrate (mg g⁻¹DW) and ammonium (mg g⁻¹DW) in barley leaves. Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**), and p/<0.001 (***).

	N organic (mg g ⁻¹ DW)			TNC (mg g ⁻¹ DW)		
	0.01 μM Zn	1 μM Zn	100 μM Zn	0.01 μM Zn	1 μM Zn	100 μM Zn
0 mM Si	7.60±0.25 ^b	22.01±0.70 ^a	10.59±0.53 ^b	10.25±0.22 ^b	23.77±0.50 ^a	11.88±0.17 ^b
1 mM Si	10.42±0.40 ^a	13.73±0.76 ^a	14.98±0.59 ^a	15.14±0.16 ^a	14.84±0.3 ^b	15.56±0.33 ^a
P-value	***	***	***	***	***	***
0.01 μM Zn		9.01 ^b			12.70 ^c	
1 μM Zn		17.87 ^a			19.30 ^a	
100 μM Zn		10.39 ^b			13.72 ^b	
P-value		***			***	
LSD_{0.05}		1.85			0.50	

Table 6 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the concentration of N organic (mg g⁻¹DW) and total nitrogen content (mg g⁻¹DW) in barley leaves. Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**), and p/<0.001 (***).

4.4.2 Effects of Zn and Si on enzymes of nitrogen metabolism

Nitrate reductase activity was not significantly affected by Zn nutrition, remaining at the value $0.30 \mu\text{M NO}_2^- \text{mg prot}^{-1} \text{min}^{-1}$. Interestingly, Si application raised nitrate reductase activity by 19% under both low and control Zn doses; while in plants under Zn toxicity a significant decrease was observed (24%) with respect to plants grown without Si.

The highest glutamine synthetase activity was observed in barley plants subjected to Zn toxicity with a value of $816 \mu\text{M glutamyl hydroxamate mg prot}^{-1} \text{min}^{-1}$ (2.63 fold greater than control value). Zn deficiency also increased GS values with respect to control plants presented a value of $517 \mu\text{M glutamyl hydroxamate mg prot}^{-1} \text{min}^{-1}$ (1.66 fold greater than control value). Conversely, Si reduced GS activity when low and high Zn doses are applied to barley plants, with a decrease of 28% for Zn deficiency and 46% for Zn toxicity. Oppositely, GS activity was higher under control conditions when Si was applied, increasing the value of 20% respect to control.

In contrast to GS, GOGAT activity was higher (+88%) in Zn deficient barley plants with respect to controls, as well as in plants subjected to Zn toxicity (+58%). Si application caused an inhibition of 20% in GOGAT activity of barley plants grown under both deficiency and toxicity and did not produce changes in the activity in control plants.

Zn toxicity caused a noticeable increase in glutamate dehydrogenase activity, tripling the value observed in control plants. A similar, but lower increase was measured in Zn deficient plants (+75%). On the other hand, it is noteworthy that Si supply increased GDH activity in plants regardless the Zn treatment considered (Fig. 6).

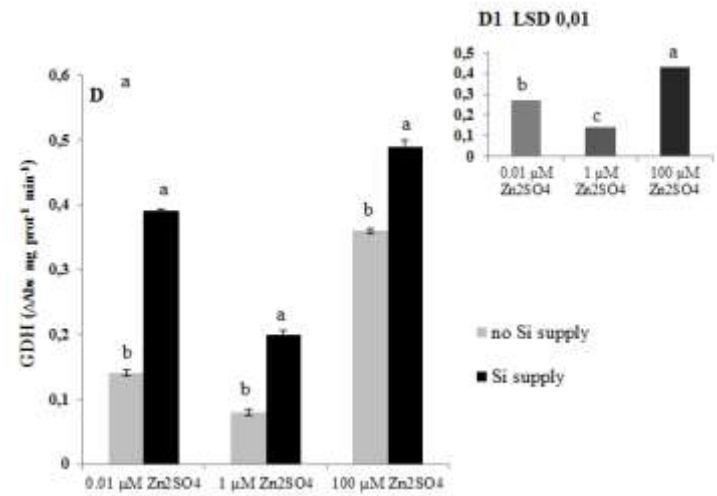
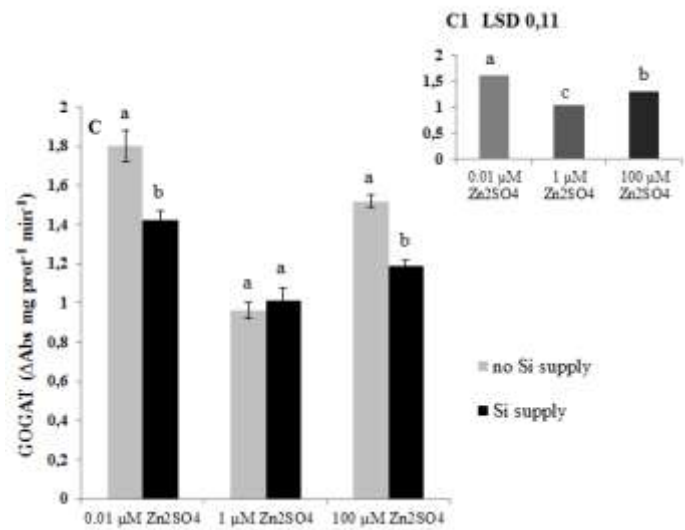
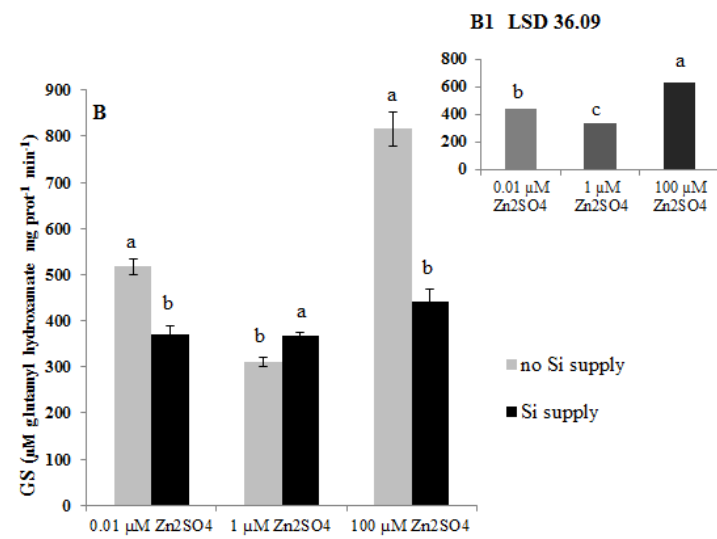
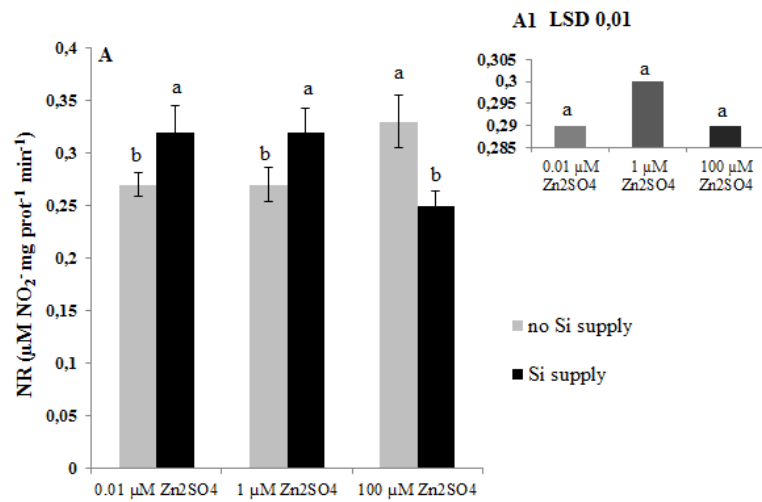


Fig. 6 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the activity of NR ($\mu\text{M NO}_2^- \text{ mg prot}^{-1} \text{ min}^{-1}$) (A), GS ($\mu\text{M glutamyl hydroxamate mg prot}^{-1} \text{ min}^{-1}$) (B), GOGAT ($\Delta\text{Abs mg prot}^{-1} \text{ min}^{-1}$) (C) and GDH ($\Delta\text{Abs mg prot}^{-1} \text{ min}^{-1}$) (D) in barley leaves.

The columns Values are mean \pm standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05). A1, B1, C1 and D1 Values are means (control +Si supply n = 18) and differences between means were compared using LSD test (P =0.05). Levels of significance are represented by NS (non-significant) P > 0.05; * P < 0.05; ** P < 0.01 and *** P < 0.001 relative to the control.

4.4.3 Aspartate aminotransferase (AAT)

Aspartate amino transferase (AAT) activity was almost double-increased by both Zn deficiency and toxicity in comparison to control plants. Furthermore, Si application in control plants increased AAT activity by 38%. In contrast, a 10% decrease was observed in plants exposed to the combined treatment of Zn deficiency and Si; no change was observed when plants were grown under Zn excess in the presence of Si (Fig. 7).

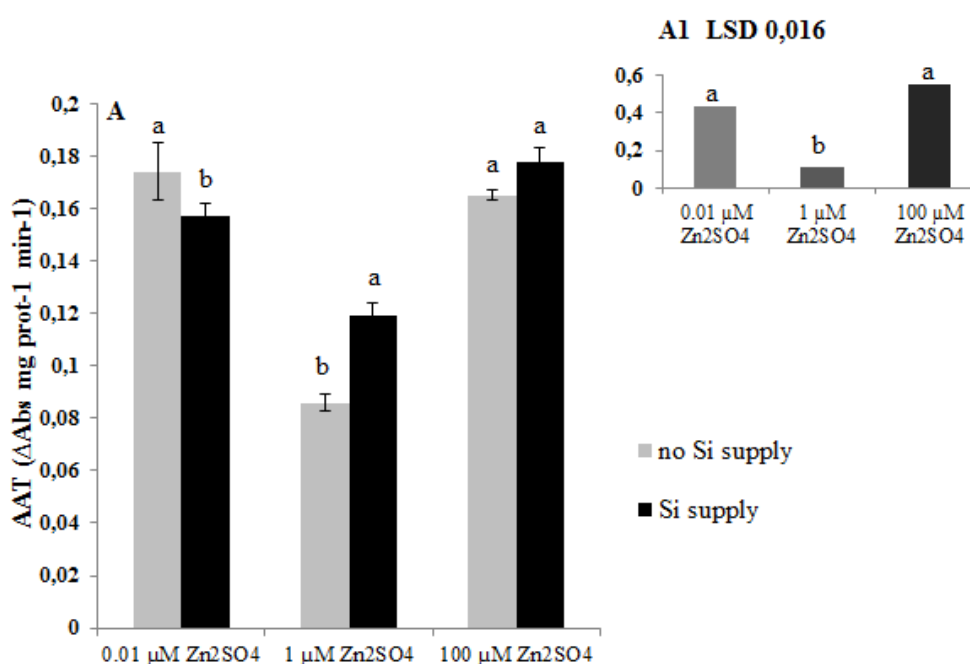


Fig. 7 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the AAT activity ($\Delta\text{Abs mg prot}^{-1} \text{ min}^{-1}$) (A) in barley leaves.

The columns Values are mean \pm standard error ($n = 9$), the differences between means were compared with the minimum significant difference of Fisher test (LSD; $P = 0.05$).

A1 Values are means (control +Si supply $n = 18$) and differences between means were compared using LSD test ($P = 0.05$). Levels of significance are represented by NS (non-significant) $P > 0.05$; * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ relative to the control.

4.4.4 Glyoxylate oxidase (GO)

Glyoxylate oxidase (GO) showed higher levels of activity under both Zn deficiency and toxicity in comparison to control conditions (+65%). Si application raised GO activity about 40% when control Zn dose was applied whereas produced an appreciable diminution in the rest of doses, particularly in Zn toxic leaves with a decrease of 35% (Fig. 8).

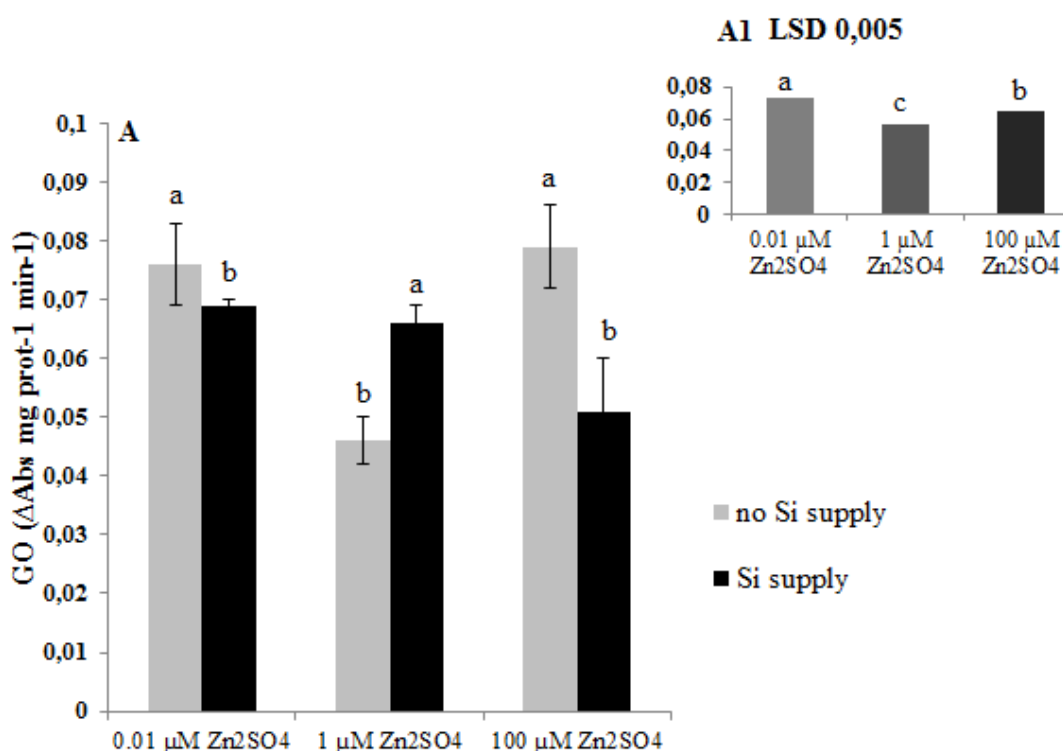


Fig. 8 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the GO activity ($\Delta\text{Abs mg prot}^{-1} \text{ min}^{-1}$) (A) in barley leaves.

The columns Values are mean \pm standard error ($n = 9$), the differences between means were compared with the minimum significant difference of Fisher test (LSD; $P = 0.05$). A1 Values are means (control +Si supply $n = 18$) and differences between means were compared using LSD test ($P = 0.05$). Levels of significance are represented by NS (non-significant) $P > 0.05$; * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ relative to the control.

4.4.5 Glutamate glyoxylate aminotransferase (GGAT)

Glutamate Glyoxylate aminotransferase (GGAT) activity was increased 5-fold increased by both Zn toxicity and Zn deficiency in comparison to control plants.

Si supply affected unequally GGAT activity depending on the Zn dose applied. Thus, in presence of Si, GGAT activity lowered in Zn deficient plants (-33%) and rose in control plants with a slight but significant increase of 33%. Differently, no change was observed in plants grown under Zn toxicity combined with Si nutrition in comparison to plants without a Si supply (Fig. 9).

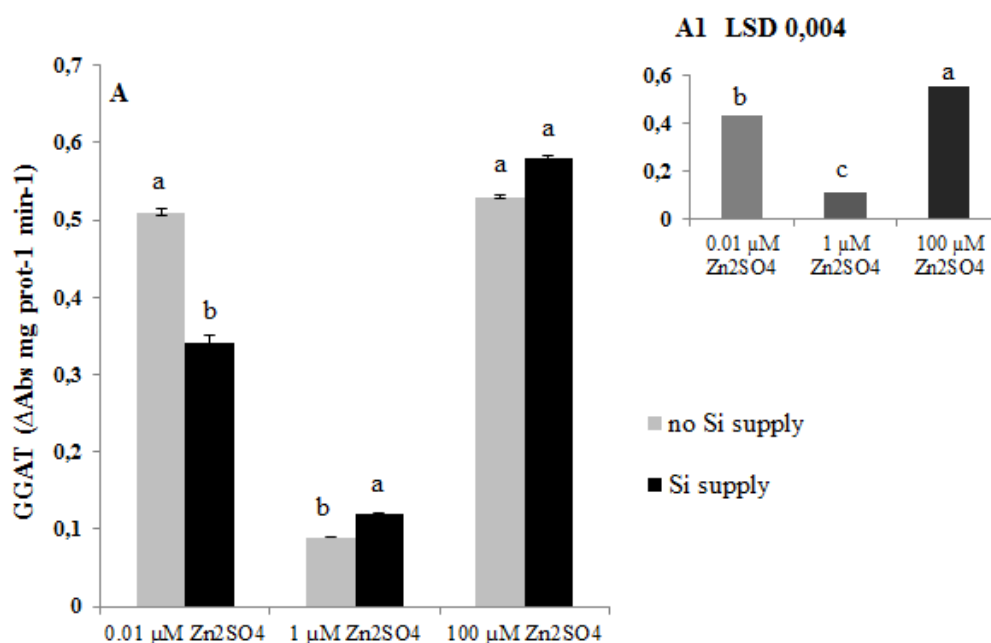


Fig. 9 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the GGAT activity ($\Delta\text{Abs mg prot}^{-1} \text{ min}^{-1}$) (A) in barley leaves.

The columns Values are mean \pm standard error ($n = 9$), the differences between means were compared with the minimum significant difference of Fisher test (LSD; $P = 0.05$).

AI Values are means (control +Si supply $n = 18$) and differences between means were compared using LSD test ($P = 0.05$). Levels of significance are represented by NS (non-significant) $P > 0.05$; * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ relative to the control.

4.5 Osmoprotectant compounds derived from N metabolism

Regarding osmotic protective compounds such as proline and glycine betaine, we observed that proline content in leaf of barley plants increased with an increase in Zn stress. The Zn-optimum plants manifested low level of proline content compared with the rest of treatments. Proline content decrease by 67% and 78% in the leaf of plants subjected to the combined treatments of Si with low and high Zn doses respectively. In Zn control plants, Si supply induced an increment of 70% of Pro content

On the other hand, glycine betaine levels were higher under Zn deficiency and toxicity with a 52% increase. Si application did not induced changes in GB content with respect to barley plants grown without Si (Fig. 10).

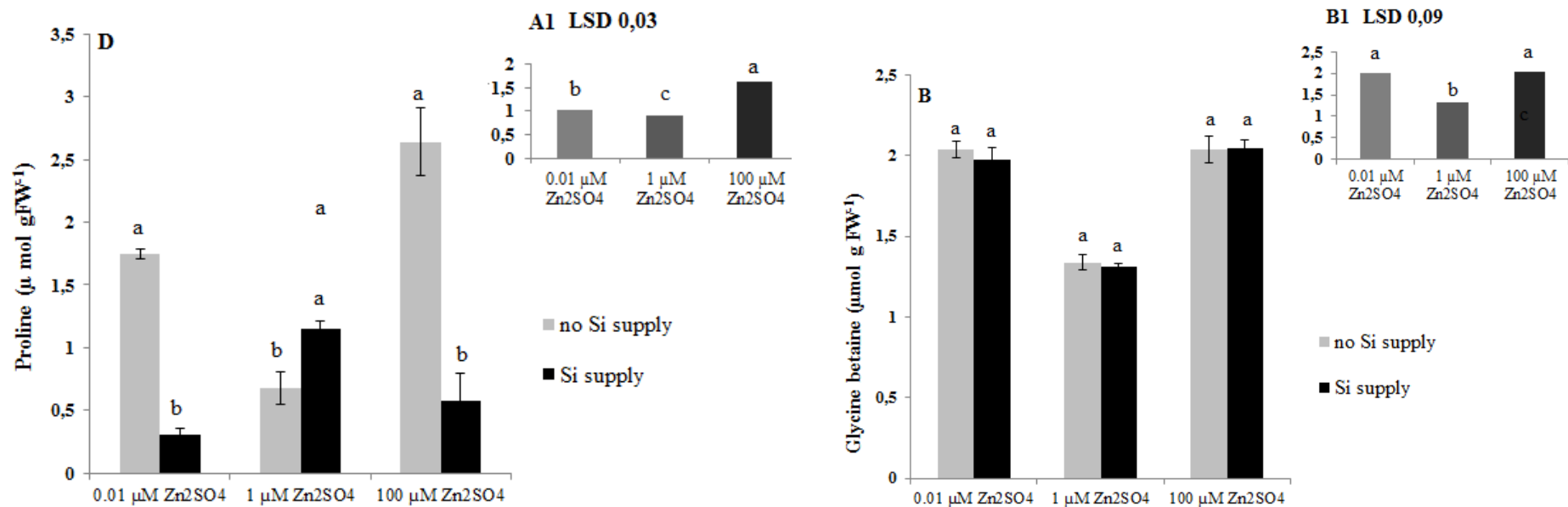


Fig. 10 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the concentration of Proline ($\mu\text{mol g FW}^{-1}$) (A) and Glycine betaine ($\mu\text{mol g FW}^{-1}$) (B) in barley leaves. The columns Values are mean \pm standard error ($n = 9$), the differences between means were compared with the minimum significant difference of Fisher test (LSD; $P = 0.05$). A1 and B1 Values are means (control +Si supply $n = 18$) and differences between means were compared using LSD test ($P = 0.05$). Levels of significance are represented by NS (non-significant) $P > 0.05$; * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ relative to the control.

4.6 Effects of Zn nutrition and Si supply on free amino acids levels

Zn deficiency and toxicity caused an increase in total free amino acids in comparison to control conditions. Si application reduced total amino acid when plants were grown under low and control Zn doses, while high Zn dose did not produce visible effects on the accumulation of these compounds.

Zn toxicity caused an increment in Glu concentration in barley plants while Zn deficiency did not affect significantly to Glu levels. Si did not affect to Glu concentration when low and high Zn doses were applied, however it decrease the levels of this amino acid when control Zn dose was applied.

Regarding Asp levels, this increased as a result of high Zn dose application while low Zn dose did not cause changes in Asp concentration. Barley plants with and without Si supply presented similar values of Asp concentration.

Barley plants presented no significant change in Arg concentration when the different Zn dose were compared. However, Si application reduced Arg accumulation in both Zn deficiency and Zn control plants and did not affect when high Zn dose was applied.

Histidine concentration was higher when high Zn dose was applied to barley plants, but His accumulation did not suffer changes when low Zn dose was applied. With respect Si, plants supplied with low and control Zn dose and Si decreased its His accumulation, while Si did not affect to His when high Zn dose was applied.

Regarding Gly accumulation, it was not affected by the different Zn supplies in barley plants. Nevertheless, Si application caused a decreased in Gly concentration in all barley plants disregarding the Zn dose applied in comparison to plants grown without Si.

With respect Ser levels, this were not significantly affected by the three different Zn supplies. In contrast, when barley plants were supplied with Si, they showed lower Ser

accumulation, regardless the Zn dose applied, in comparison to control plants without Si.

High Zn supplied increased Thr accumulation in barley plants in comparison to plants grown under control Zn dose, whereas Zn deficiency did not affect to the accumulation of this amino acid. Likewise, Si application did not cause effects on Thr accumulation in all barley plants employed in the experiment.

Zn deficiency caused an increment in Tyr concentration with respect to control Zn plants. Zn toxicity did not produce changes in Tyr accumulation. On the hand Si application only enhanced Tyr accumulation when control Zn dose was applied, while in the rest of Zn doses no changes were observed.

Pro accumulation was promoted in plants supplied with high Zn dose and was not affected by Zn deficiency in comparison to control plants. On the one hand, Si caused an increase in Pro accumulation in Zn deficient plants, on the other hand, control and high Zn doses reduced Pro accumulation in comparison of plants grown without Si.

Cys levels were not significantly affected by Zn supply. Nevertheless, when Si was added to the nutritive solution, barley plants registered a decrease in Cys accumulation disregarding the Zn supply in comparison to plants that grew without Si in the nutritive solution.

Ala concentration was significantly higher in plants treated with high Zn dose in comparison to plants treated with low Zn dose, however Ala levels in both treatments were no significantly different to those of control Zn dose. On the other hand, Si had no effect on Ala accumulation regardless the Zn dose applied.

With respect to Lys, this amino acid was more accumulated in plants treated with high Zn dose in comparison to control plants. Zn deficiency had no effect on the accumulation of Lys. Si application only reduced Lys levels in plants treated with

control Zn dose, whereas in the rest of Zn doses did not change the accumulation of this amino acid.

Zn toxicity produced an increase in Val levels in comparison to control Zn dose, while Zn deficiency did not produce effects on this amino acid. The supply of Si to the nutrient solution caused the increase in Val levels when was combined with low and control Zn doses, however did not change the concentration of this amino acid when high Zn dose was applied.

Regarding Leu, its accumulation was greater in plants subjected to Zn toxicity in compared to control plants but Leu accumulation was not different under Zn deficiency. Likewise, Si application did not produce effect on Leu concentration disregarding de Zn dose applied.

Barley plants presented similar Ile values disregarding the Zn dose applied. In contrast, Si application enhanced Ile accumulation but only in plants treated with Zn deficiency. In the rest of Zn doses, Si did not produce significant effects on Ile levels.

Both Zn deficiency and toxicity increased Phe concentration in comparison to control Zn dose. However, high Zn dose produced a higher increment in Phe levels. In addition, Si supply to the nutritive solution increased Phe accumulation in plants subjected to low and control Zn doses, although did not produce change in plants subjected to Zn toxicity (Table 7).

	ASP	ASP	GLU	GLU	GLY	GLY	SER	SER	ALA	ALA	LEU	LEU	PRO	PRO	CYS	CYS	ARG	ARG
	se		se		se		se		se		se		se		se		Se	
0,01 μ M Zn	4.87	0.52	2.51	0.33	0.40	0.04	5.18	0.63	2.46	0.33	0.64	0.19	0.95	0.04	0.70	0.01	6.57	0.59
0,01 μ M Zn+Si	4.11	0.24	1.03	0.06	0.29	0.02	3.48	0.11	1.98	0.04	0.73	0.02	0.31	0.05	0.32	0.02	5.62	0.23
1 μ M Zn	4.24	0.42	2.73	0.17	0.47	0.01	4.59	0.59	2.73	0.18	0.76	0.13	0.68	0.13	0.76	0.07	8.56	1.04
1 μ M Zn+Si	4.21	0.08	2.59	0.06	0.35	0.05	3.38	0.01	1.39	0.01	0.86	0.01	1.15	0.06	0.29	0.01	3.67	0.03
100 μ M Zn	5.82	0.55	2.95	0.29	0.46	0.04	7.54	0.53	2.98	0.36	1.27	0.27	2.64	0.27	0.90	0.08	7.61	1.07
100 μ M Zn + Si	4.55	0.84	2.08	0.42	0.37	0.01	3.98	0.95	2.21	0.37	1.21	0.29	0.58	0.21	0.44	0.09	5.38	0.62

Table 7 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the concentration of free amino acids in barley leaves. Amino acid levels are expressed as μ ml/gFW \pm Standard error (n=9).

4.7 Photosynthetic pigments and SPAD value

Photosynthetic pigments levels were significantly affected by Zn nutrition regime. In barley leaves, low Zn concentrations induced a slight but significant decrease of chlorophyll a content with respect to control. Similarly, Zn toxicity determined an even greater decrease (-14%) in chl a. When Si was added, chlorophyll a content significantly increased by 10% in both Zn deficient and control plants. Differently, Si supply in combination with a toxic concentration of Zn did not provoke any appreciable variation. Zn deficiency induced a slight and significant diminution of chlorophyll b content; moreover, plants treated with Zn excess presented a noticeable decrease of this pigment (+12%). Si supply caused an increase of chl b content both in control and Zn deficient leaves (+12%); under Zn toxicity condition Si supplementation was ineffective.

The results for chlorophyll a / chlorophyll b ratio (Chla/Chlb) showed no significant differences when the different Zn dose were compared. However, when Si was applied to the liquid growth medium with high Zn dose, the values of Chla/Chlb were higher than in plants without Si application. In the rest of Zn doses Si did not cause significant effects on Chla/Chlb.

Very different was the response to Zn stress and Si application on carotenoids content. Zn deficiency increase of about 30% carotenoids concentration respect to control plants; differently, in leaves treated with Zn excess, carotenoids level remained stable to control levels. After Si application, Zn deficient leaves did not show any change in the pigment content respect to control, and an appreciable and significant increase of carotenoids level was observed in both control (+28%) and Zn toxic (+15%) plants.

The SPAD value of barley leaves showed a similar change respect to control when Zn was supply in deficient or toxic concentrations. Both treatments induced a severe decrease in SPAD values (+40%).

Silicon supply stabilized SPAD levels substantially to control plants levels (Table 8).

	CHLOROPHYLL A (mg gFW ⁻¹)			CHLOROPHYLL B (mg gFW ⁻¹)			CHLA/CHLB		
	0.01 μM Zn	1 μM Zn	100 μM Zn	0.01 μM Zn	1 μM Zn	100μM Zn	0.01 μM Zn	1 μM Zn	100 μM Zn
0 mM Si	0.187±0.002 ^b	0.191±0.002 ^b	0.163±0.002 ^b	0.082±0,002 ^b	0.084±0.001 ^b	0.074±0.001 ^a	2.265±0,014 ^a	2.272±0.036 ^a	2.195±0.021 ^b
1 mM Si	0.203 ±0.001 ^a	0.201±0.002 ^a	0.164±0.005 ^a	0.093±0.001 ^a	0.094±0.001 ^a	0.072±0.001 ^a	2.237±0.017 ^a	2.225±0.012 ^a	2.313±0.021 ^a
P-valor	***	***	NS	***	***	NS	NS	NS	**
0.01 μM Zn		0.195 ^b			0,088 ^b			2.25 ^a	
1 μM Zn		0.201 ^a			0,093 ^a			2.25 ^a	
100 μM Zn		0.166 ^c			0,081 ^c			2.25 ^a	
P-value		***			***			NS	
LSD_{0.05}		0.003			0,002			0,04	

	CAROTENOIDS (mg gFW ⁻¹)				SPAD CHLOROPHYLL	
	0.01 μM Zn	1 μM Zn	0.01 μM Zn	0.01M Zn	1 μM Zn	100 μM Zn
0 mM Si	0.027±0.001 ^a	0.021±0.001 ^b	0.027±0.001 ^a	26.63±0.44 ^b	44.40±0.41 ^a	27.42±0.43 ^b
1 mM Si	0.027±0.001 ^a	0.027±0.001 ^a	0.027±0.001 ^a	43.27±0.17 ^a	43.73±0.24 ^a	37.71±0.31 ^a
P-value	NS	***	NS	***	NS	***
0.01 μM Zn		0.088 ^b			34.95 ^b	
1 μM Zn		0.093 ^a			44.08 ^a	
100 μM Zn		0.081 ^c			32.56 ^c	
P-value		***			***	
LSD_{0.05}		0.001			0,57	

Table 8 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the concentration of leaf pigments (mg g⁻¹FW) and SPAD index in barley leaves. Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**), and p/<0.001 (***).

4.8 Effects of Zn nutrition and Si supply on photosynthetic parameters

The analysis of Chla fluorescence revealed different results depending on the Zn applied and whether Si was applied or not to plants. Thus, Zn dose did not affect significantly to F_o parameter. However, Si application reduced F_o value under Zn deficiency and increased it slightly in plants that received control and high Zn doses in comparison to plants without Si.

F_m values were not affected by either Zn doses, although it did under Si supply. Thus, under Zn deficiency, Si increased F_m value and under Zn control dose decreased its value. Si had no effect on F_m parameter under Zn toxicity.

Zn supply did affect to F_v values in barley plants. We observed that F_v values were lower in plants subjected to Zn stress: we did not find significant differences between low and high Zn doses. On the other hand, Si application enhanced F_v value in plants grown under both Zn deficiency and toxicity, whereas it has no effect in plants grown under Zn control dose.

Barley presented similar F_v/F_m values regardless the Zn dose applied. Nevertheless, Si application caused the increment in F_v/F_m value when plants were grown under both low and high Zn doses. Under control Zn dose, we did not observe any effect of Si in F_v/F_m value in comparison to plants without Si.

PI_{ABS} , an important fluorescence parameter indicative of plant vitality, was not affected by the different Zn doses. Si induced higher PI_{ABS} under all Zn doses applied, in comparison to control plants without Si.

RC/ABS , a parameter related to the active ratio of photosynthetic reaction centres, remained unchanged under different Zn nutrition regimes, and Si application enhanced RC/ABS values regardless the Zn supply in comparison to control plants.

Regarding parameters relative to electron flux within photosystem II, differences were recorded depending on the Zn dose applied. Therefore, Ψ_{E_0} values were lower in plants grown under both Zn deficiency and toxicity in comparison to control plants. In contrast, Si application enhanced Ψ_{E_0} regardless the Zn dose applied.

Another parameter relative to electron flux, Φ_{E_0} , showed a decrease caused by high Zn dose, while low Zn dose did not affect Φ_{E_0} in comparison to control Zn dose. Si application also increased this electron flux parameter disregarding the Zn dose applied. The time to reach maximum fluorescence (tFm) was negatively affected in plants subjected to Zn deficiency and particularly in plants grown under Zn toxicity that exhibited the lowest tFm values in comparison to control plants. However, Si application enhanced tFm values significantly with respect to plants grown without Si and regardless the Zn dose.

K step is a parameter indicative of an uncoupling of electron flux between oxygen evolving centre and the PSII core. No significant difference was observed among plants grown under the different Zn regimes. In addition, Si application decreased K step value under all Zn regimes in comparison to plants without Si.

The Area value above the fluorescence transient curve was lower in plants grown under both deficiency and toxicity conditions in comparison to control Zn dose. However, Si supply enhanced Area values of all barley plants employed in the experiment.

Sm value was negatively affected in plants grown under Zn deficiency and Zn toxicity, that presented lower values than plants grown under control Zn dose. Furthermore, plants supplied with Si registered higher Sm values than plants without Si supply.

The number of times that quinone A (Q_A) is reduced from time 0 to the time when maximum fluorescence is reached is the N parameter. Both Zn deficiency and Zn toxicity affected negatively the N parameter although to a greater extent by Zn

deficiency. Nevertheless, Si increased N values disregarding the Zn regime compared with control plants.

Regarding the parameters related to electron fluxes through the diverse parts of the PSII, we observed uneven results depending on the Zn dose and Si application. For instance, Zn deficient plants presented the lowest ABS/RC values, followed by plants grown under Zn toxicity that presented lower ABS/RC than control Zn plants. Si application decreased ABS/RC parameter in Zn deficient plants, whereas Si increased ABS/RC in plants grown under control and high Zn dose.

Plants grown under Zn deficiency registered lower TC/RC value while this was not affected by Zn toxicity in comparison to plants grown under control Zn dose. In addition, plants supplied with Si showed lower TR/RC value when were grown under both Zn deficiency and toxicity but not under Zn control dose because they presented higher TR/RC than plants grown without Si.

Finally, ET/RC parameter was lower in plants grown under both low and high Zn dose in comparison to control Zn plants. In Zn deficient plants Si application did not cause any effect on ET/RC value. However, Si did increase ET/RC in plants grown under control Zn and high Zn doses (Table 9 to 12; Fig. 11).

	<i>tFm</i>			<i>F0</i>			<i>Fm</i>			<i>Fv</i>		
	0.01µM Zn	1µM Zn	100µM Zn	0.01µM Zn	1µM Zn	100µM Zn	0.01µM Zn	1µM Zn	100µM Zn	0.01µM Zn	1µM Zn	100µM Zn
0 mM Si	278 ^b	293 ^b	231 ^b	498 ^a	493 ^b	473 ^a	2478 ^b	2738 ^a	2535 ^a	2041 ^b	2203 ^a	2022 ^b
1 mM Si	328 ^a	725 ^a	600 ^a	483 ^b	514 ^a	498 ^a	2681 ^a	2516 ^b	2476 ^a	2256 ^a	2204 ^a	2212 ^a
p-value	*	***	***	***	**	NS	*	*	NS	***	NS	***
0.01 µM Zn		303 ^c			491 ^a			2579 ^a			2148 ^{ab}	
1 µM Zn		509 ^a			503 ^a			2627 ^a			2204 ^a	
100 µM Zn		415 ^b			486 ^a			2555 ^a			2117 ^b	
p-value		***			NS			NS			*	
LSD_{0.05}		41			20			117			57	

Table 9 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply on total Fm (*tFm*), *F0*, *Fm* and *Fv* in barley leaves. A complete description of parameters is given in the text. Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**), and p/<0.001 (***).

	<i>Fv/Fm</i>			Ψ_{Eo}			Φ_{Eo}			<i>PI_{ABS}</i>		
	0.01 μ M Zn	1 μ M Zn	100 μ M Zn	0.01 μ M Zn	1 μ M Zn	100 μ M Zn	0.01 μ M Zn	1 μ M Zn	100 μ M Zn	0.01 μ M Zn	1 μ M Zn	100 μ M Zn
0 mM Si	0.81 ^b	0.82 ^a	0.80 ^b	0.44 ^b	0.47 ^b	0.43 ^b	0.38 ^b	0.39 ^b	0.35 ^b	1.39 ^b	1.45 ^b	1.32 ^b
1 mM Si	0.82 ^a	0.81 ^a	0.82 ^a	0.53 ^a	0.56 ^a	0.52 ^a	0.43 ^a	0.41 ^a	0.43 ^a	1.99 ^a	1.95 ^a	2.01 ^a
p-value	*	NS	**	***	***	***	***	***	***	***	**	***
0.01 μM Zn		0.82 ^a			0.49 ^b			0.41 ^a			1.69 ^a	
1 μM Zn		0.82 ^a			0.52 ^a			0.40 ^{ab}			1.70 ^a	
100 μM Zn		0.81 ^a			0.48 ^b			0.39 ^b			1.67 ^a	
p-value		NS			***			*			NS	
LSD_{0.05}		0.01			0.01			0.01			0.16	

Table 10 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply on *Fv/Fm*, Ψ_{Eo} , Φ_{Eo} and *PI_{ABS}* in barley leaves.

A complete description of parameters is given in the text. Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**), and p<0.001 (***).

	<i>K step</i>			<i>RC/ABS</i>			<i>Area</i>			<i>Sm</i>		
	0.01 μ M Zn	1 μ M Zn	100 μ M Zn	0.01 μ M Zn	1 μ M Zn	100 μ M Zn	0.01 μ M Zn	1 μ M Zn	100 μ M Zn	0.01 μ M Zn	1 μ M Zn	100 μ M Zn
0 mM Si	1279 ^a	1257 ^a	1293 ^a	0.34 ^b	0.37 ^b	0.33 ^b	37542 ^b	52570 ^b	35108 ^b	20.86 ^b	23.38 ^b	17.01 ^b
1 mM Si	1119 ^b	1141 ^b	1137 ^b	0.42 ^a	0.40 ^a	0.41 ^a	57329 ^a	87361 ^a	60933 ^a	26.28 ^a	46.21 ^a	25.33 ^a
p-value	***	***	**	***	**	***	***	***	***	***	***	**
0.01 μM Zn		1199 ^a			0.38 ^a			47435 ^b			23.57 ^b	
1 μM Zn		1199 ^a			0.38 ^a			69965 ^a			34.79 ^a	
100 μM Zn		1215 ^a			0.37 ^a			48020 ^b			21.17 ^b	
p-value		NS			NS			***			***	
LSD_{0.05}		41			0.01			5204			2.44	

Table 11 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply on *K step*, *RC/ABS*, *area* and *Sm* in barley leaves. A complete description of parameters is given in the text. Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**), and p/<0.001 (***).

	<i>ABS/RC</i>			<i>TR/RC</i>			<i>ET/RC</i>			<i>N</i>		
	0.01 $\mu\text{M Zn}$	1 $\mu\text{M Zn}$	100 $\mu\text{M Zn}$	0.01 $\mu\text{M Zn}$	1 $\mu\text{M Zn}$	100 $\mu\text{M Zn}$	0.01 $\mu\text{M Zn}$	1 $\mu\text{M Zn}$	100 $\mu\text{M Zn}$	0.01 $\mu\text{M Zn}$	1 $\mu\text{M Zn}$	100 $\mu\text{M Zn}$
0 mM Si	2.58 ^a	2.53 ^b	2.64 ^b	2.07 ^a	2.12 ^b	2.17 ^a	1.02 ^a	1.10 ^b	1.02 ^b	38.50 ^b	49.72 ^b	42.31 ^b
1 mM Si	2.46 ^b	3.57 ^a	3.06 ^a	2.02 ^b	2.49 ^a	2.37 ^b	1.05 ^a	1.56 ^a	1.24 ^a	56.08 ^a	99.99 ^a	80.60 ^a
p-value	***	***	***	***	***	***	NS	***	***	***	***	***
0.01 $\mu\text{M Zn}$		2.52 ^c			2.05 ^b			1.04 ^c			49.19 ^c	
1 $\mu\text{M Zn}$		3.05 ^a			2.30 ^a			1.33 ^a			73.20 ^a	
100 $\mu\text{M Zn}$		2.85 ^b			2.27 ^a			1.13 ^b			59.55 ^b	
p-value		***			*			***			***	
LSD_{0.05}		0.10			0.06			0.04			6.47	

Table 12 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply on *ABS/RC*, *TR/RC*, *ET/RC* and *N* in barley leaves. A complete description of parameters is given in the text. Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**), and p<0.001 (***).

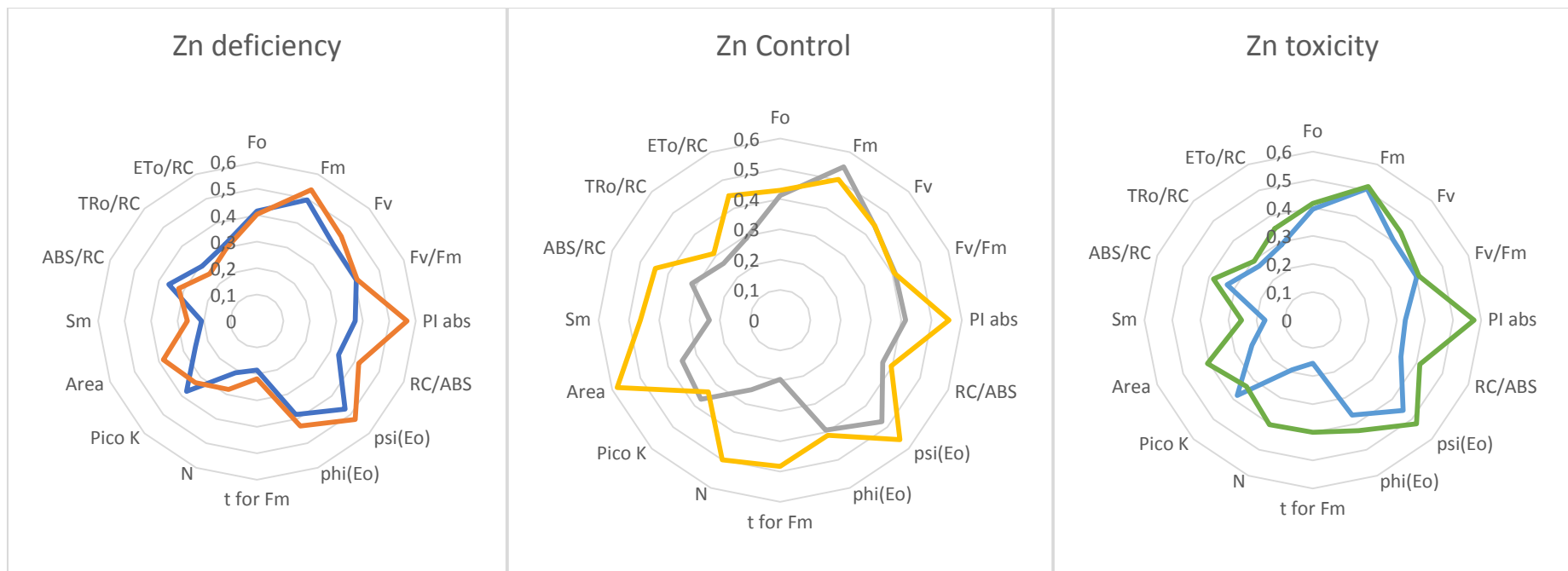


Fig. 11 Values of Chl a fluorescence parameters derived from the JIP test in barley leaves submitted to different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply. Different color lines represent different treatments: light blue, grey and blue lines represent treatments without Si; red, yellow and green lines represent treatment with 1 mM Si supply. Values are expressed as means \pm standard error (n=9).

4.9 Effects of Zn nutrition and Si supply on oxidative stress

4.9.1 Ascorbate

Zinc limitation resulted in a slight but significant decrease of 13% in reduced ascorbic acid compared with control conditions. Differently, when exposed to toxic concentrations of Zn, plants suffer an appreciable variation of 64% in the reduction of the ascorbic acid respect to plants grown under control conditions.

Adding Si to toxic Zn solution, plants did not show any changes in reduced ascorbic acid pool. Si application induced variation in plants treated with Zn deficient and control concentrations. Respect to the plants grown in absence of Si, Si treated plants increased the rate of reduction of ascorbic acid in Zn deprived condition (+9%), while decreased the rate in control condition (-15%).

Diversely, oxidized form of ascorbic acid significantly increased by 25% in both condition of Zn stress. When Si was added, plants grown under control conditions did not show any variation in the content of the oxidized form, while a severe diminution of DHA was recorded in barley leaves exposed to deficient and toxic Zn concentrations, particularly in Zn limited plants (+38%).

The ratio reduced/oxidized ascorbic acid suffered a similar decrease of 32% in Zn limited and toxic plants compared to control. Si supply induced a slight but significant decrease of 17% in the rate of 1 μM ZnSO_4 plants. On the contrary, in presence of Si, the ratio *ASA/DHA* considerably increased under Zn deficiency, doubling control values, and a lower but equally significant increment (+27%) was observed in Zn toxicity treatment.

In comparison to control conditions, limitation and overexposure of Zn negatively affected the redox state of the vegetable cells inducing a diminution of the rate similar in the both stress expositions (-9%). Si application reversely changed the redox state to that of plants growth without Si: the higher levels were observed under Zn toxicity (+7%) and Zn limitation (15%). Under control conditions, Si induced a slight but significant diminution of the rate (-5%) (Table 13).

		AsA ($\mu\text{g g}^{-1}\text{FW}$)	DHA ($\mu\text{g g}^{-1}\text{FW}$)	AsA/DHA	Redox status
0.01 μM Zn	0 mM Si	203 ^b	93.07 ^a	2.21 ^b	68.65 ^b
	1 mM Si	221 ^a	58.01 ^b	4.64 ^a	79.15 ^a
	p-value	*	***	***	***
1 μM Zn	0 mM Si	234 ^a	75.21 ^a	3.24 ^a	76.39 ^a
	1 mM Si	198 ^a	74.58 ^a	2.70 ^b	72.77 ^b
	p-value	***	NS	***	***
100 μM Zn	0 mM Si	219	94.87 ^a	2.32 ^b	69.80 ^b
	1 mM Si	221	75.11 ^b	2.95 ^a	74.68 ^a
	p-value	NS	***	***	***
0.01 μM Zn		212 ^a	75.54 ^b	3.55 ^a	75.08 ^a
1 μM Zn		216 ^{ab}	74.90 ^b	2.97 ^b	74.58 ^a
100 μM Zn		220 ^a	84.99 ^a	2.64 ^c	72.24 ^b
P-value		NS	**	***	**
LSD_{0.05}		0.06	4.63	0.14	1.36

Table 13 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the ASA content ($\mu\text{g g}^{-1}\text{FW}$), DHA content ($\mu\text{g g}^{-1}\text{FW}$), ratio ASA/DHA and ascorbate state redox in barley leaves.

Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by $p>0.05$: NS (not significant), $p<0.05$ (*), $p<0.01$ (**) and $p/<0.001$ (***).

4.9.2 Ascorbate peroxidase

Barley plants exposed to Zn deficiency did not show a significant variation in ascorbate peroxidase activity compared to controls. The same behavior was observed in plants treated with the nutrient solution containing toxic concentration of Zn. Differently, when Si was added to the plant nutrition, the enzyme activity severely decreased in all the nutrient conditions with a more reduction in control (-41%) and toxic (-25%) conditions (Fig. 12).

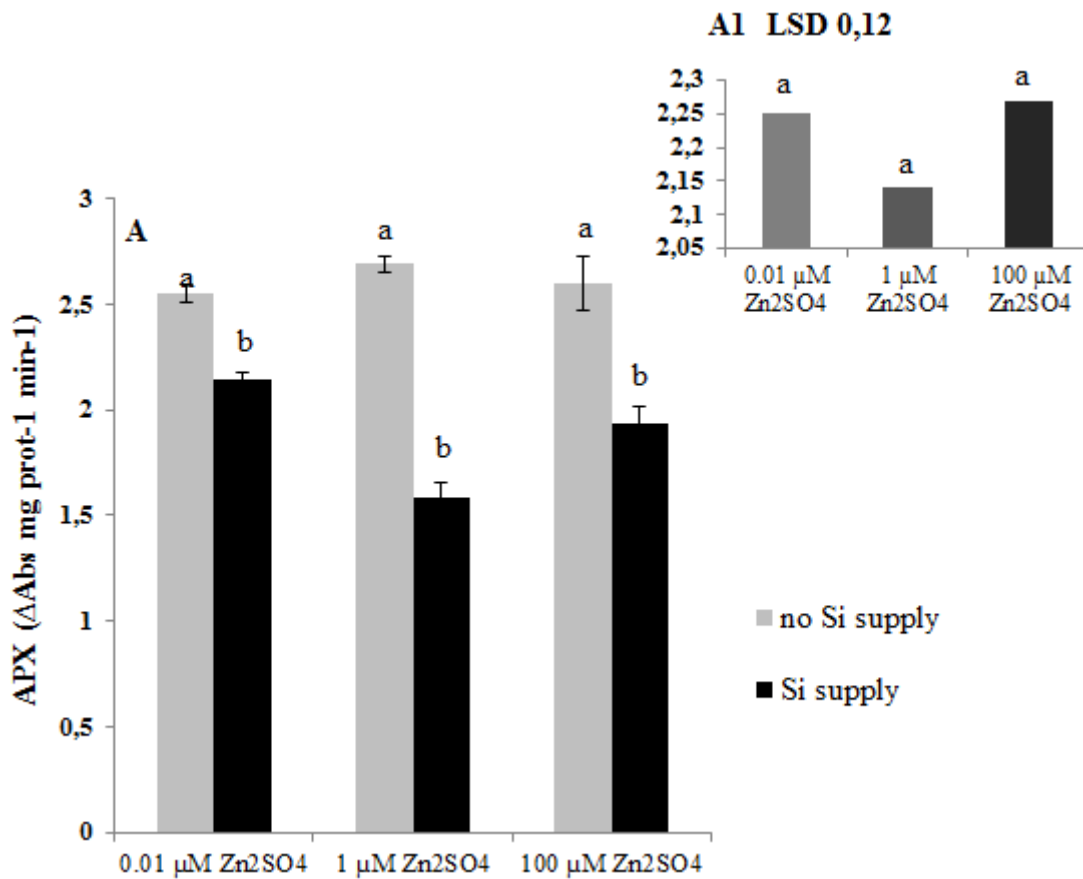


Fig. 12 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the glutathione reductase activity ($\Delta\text{Abs mg prot}^{-1} \text{ min}^{-1}$) (A) in barley leaves. Values are mean \pm standard error ($n = 9$), the differences between means were compared with the minimum significant difference of Fisher test (LSD; $P = 0.05$). A1 Values are means (control +Si supply $n = 18$) and differences between means were compared using LSD test ($P = 0.05$). Levels of significance are represented by NS (non-significant) $P > 0.05$; * $P < 0.05$; ** $P < 0.01$ and *** $P < 0.001$ relative to the control.

4.9.3 Glutathione

The reduced form of glutathione was negatively affected by Zn deficiency, decreasing by 30% with respect to control. Similarly, this effect was appreciated in plants treated with Zn toxic concentration, where a 15% of reduction was observed. Intriguingly, GSH content was increased by Si under both Zn stress condition tested, with a slight greater increment in deficient plants (+75%) respect to toxic ones (+60%). Only under Zn control treatment, Si supplementation was ineffective.

Zn deficiency induced an increase in oxidized glutathione in barley leaves. A similar but greater response was observed in plants grown in Zn toxic conditions, where plants showed an increase of 50% respect to control rate. Anyway, in all the conditions tested, Si induced a stabilization of GSSG content to levels.

The ratio GSH/GSSG was negatively affected by Zn deficiency and toxicity, confirming the results indicated above. In contrast, when Si was added to liquid growth medium, the ratio increased its values under all treatments studied, including control. These effects are particularly noticeable under Zn deficiency conditions (+174%).

The redox state of glutathione was negatively affected by nutrition with no optimal Zn concentrations (-24%). Interestingly, Si induced an increase – about 44%- of redox state both in Zn deficient than Zn toxic leaves; only under control concentration of Zn, Si induced a not significant diminution resulting ineffective for the treatment (Table 14).

			GSH (mg g ⁻¹ FW)	GSSG (mg g ⁻¹ FW)	GSH/GSSG	Redox status
0.01 Zn	μM	0 mM Si	0.055 ^b	0.074 ^a	0.63 ^b	43.22 ^b
		1 mM Si	0.097 ^a	0.060 ^b	1.73 ^a	61.59 ^a
		p-value	***	***	***	***
1 μM Zn		0 mM Si	0.078 ^a	0.059 ^a	0.91 ^b	56.66 ^a
		1 mM Si	0.071 ^a	0.060 ^a	1.31 ^a	53.82 ^a
		p-value	NS	NS	*	NS
100 μM Zn		0 mM Si	0.066 ^b	0.089 ^a	0.73 ^b	42.61 ^b
		1 mM Si	0.106 ^a	0.064 ^b	1.06 ^a	62.01 ^a
		p-value	***	***	***	***
0.01 Zn	μM		0.076 ^a	0.067 ^b	1.18 ^a	52.41 ^b
		1 μM Zn	0.075 ^a	0.060 ^c	1.11 ^a	55.24 ^a
		100 μM Zn	0.086 ^b	0.077 ^a	0.89 ^b	52.31 ^b
		P-value	**	***	***	*
		LSD_{0.05}	0.006	0.002	0.09	2.03

Table 14 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the ASA content ($\mu\text{g g}^{-1}\text{FW}$), DHA content ($\mu\text{g g}^{-1}\text{FW}$), ratio ASA/DHA and ascorbate state redox in barley leaves.

Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by p>0.05: NS (not significant), p<0.05 (*), p<0.01 (**), and p<0.001 (***).

4.9.4 Glutathione reductase

Glutathione reductase significantly increased its activity in both Zn stress condition, with an increase of 25% in deficiency and 50% in toxicity. On the other hand, Si addition to nutrient solution did not cause any change compared to plants treated without Si in all Zn conditions tested, including control (Fig. 13).

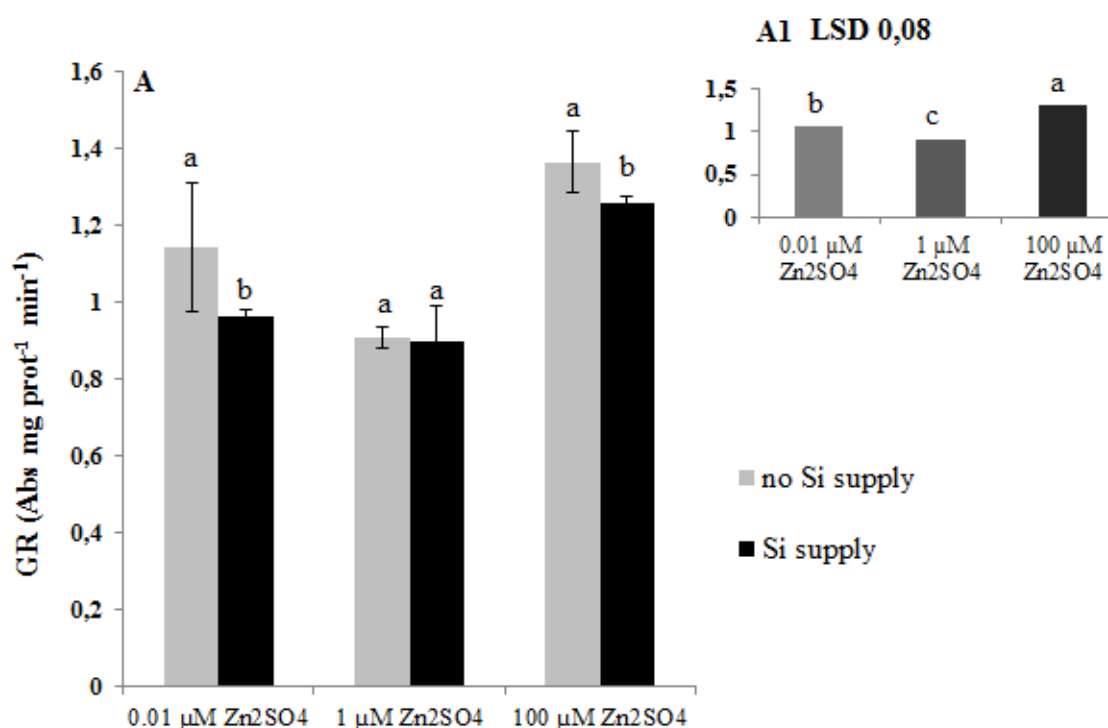


Fig. 13 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the glutathione reductase activity (Δ Abs mg prot⁻¹ min⁻¹) (A) in barley leaves.

The columns Values are mean \pm standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05).

A1 Values are means (control +Si supply n = 18) and differences between means were compared using LSD test (P =0.05). Levels of significance are represented by NS (non-significant) P > 0.05; * P < 0.05; ** P < 0.01 and *** P < 0.001 relative to the control.

4.9.5 Catalase activity

Zn deficiency induced a noticeable increase of 53% in catalase activity in barley leaves extracts. The same response, with a lower but significant entity, was observed in plants grown in Zn toxic conditions, where plants showed an increment of 35% respect to control rate. In Zn deficient plants, Si induced an appreciable diminution of the enzyme activity, bringing it almost to control level. Contrarily, the addition of Si to Zn control nutrition increased slightly but significantly the catalase activity respect to control plants. Finally, our results showed that Si was totally unaffected on plants exposed to Zn toxicity (Fig. 14).

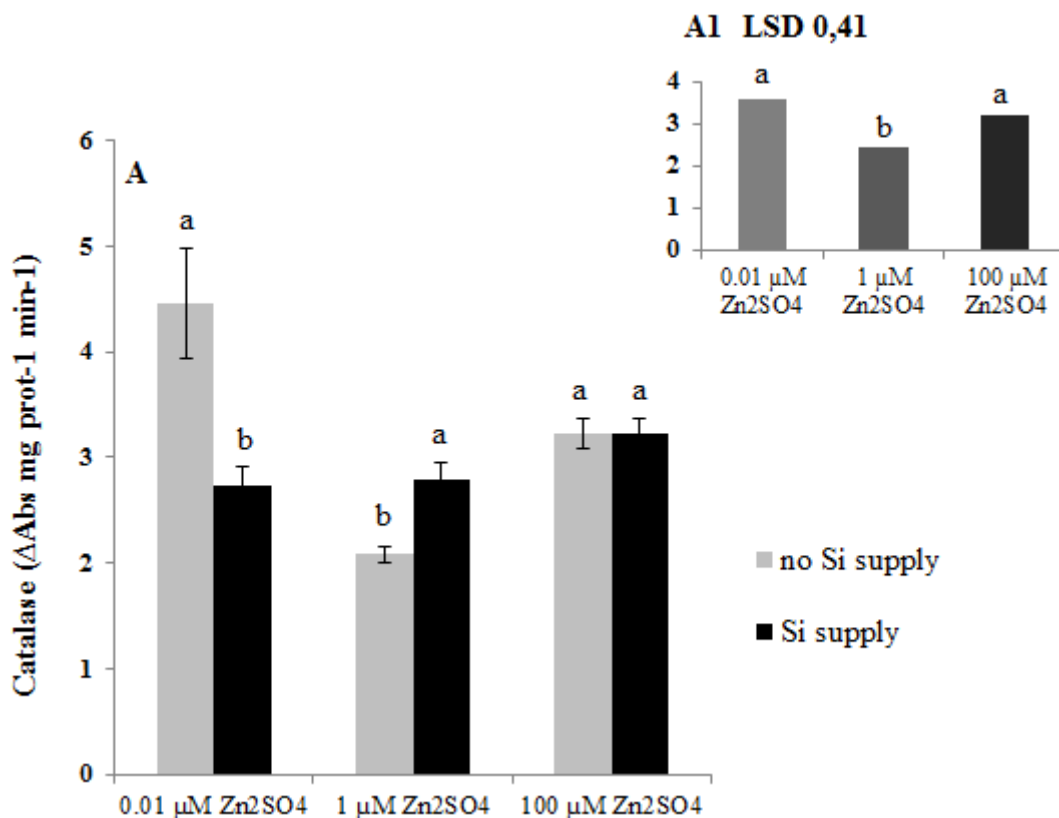


Fig. 14 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the catalase activity (Δ Abs mg prot-1 min-1) (A) in barley leaves.

The columns Values are mean \pm standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05).

A1 Values are means (control +Si supply n = 18) and differences between means were compared using LSD test (P =0.05). Levels of significance are represented by NS (non-significant) P > 0.05; * P < 0.05; ** P < 0.01 and *** P < 0.001 relative to the control.

4.9.6 Levels of indicators of oxidative stress (H_2O_2 and O_2^-)

A not-optimal Zn nutrition induced a huge increase in the levels of H_2O_2 in barley leaves, specifically an increment of 63.6% in Zn deficiency and 75.8% in Zn toxicity. Si supply reversed these effects: in Zn stress plants, H_2O_2 content lowered to control levels; differently, in Zn control plants a slight but significant increase (24%) of H_2O_2 content was recorded.

Zn deficiency induced a slight but significant increase of superoxide anion concentration (9.7%). A similar response was observed in plants grown under Zn toxic conditions, where plants showed an increment of 15%. Si application reduced of 18% superoxide anion concentration in Zn deficient barley plants and of 38.7% in Zn toxic barley plants. Different was the response in leaves grown in Zn control condition where superoxide anion showed an increase of 21% of its concentration (Fig. 15).

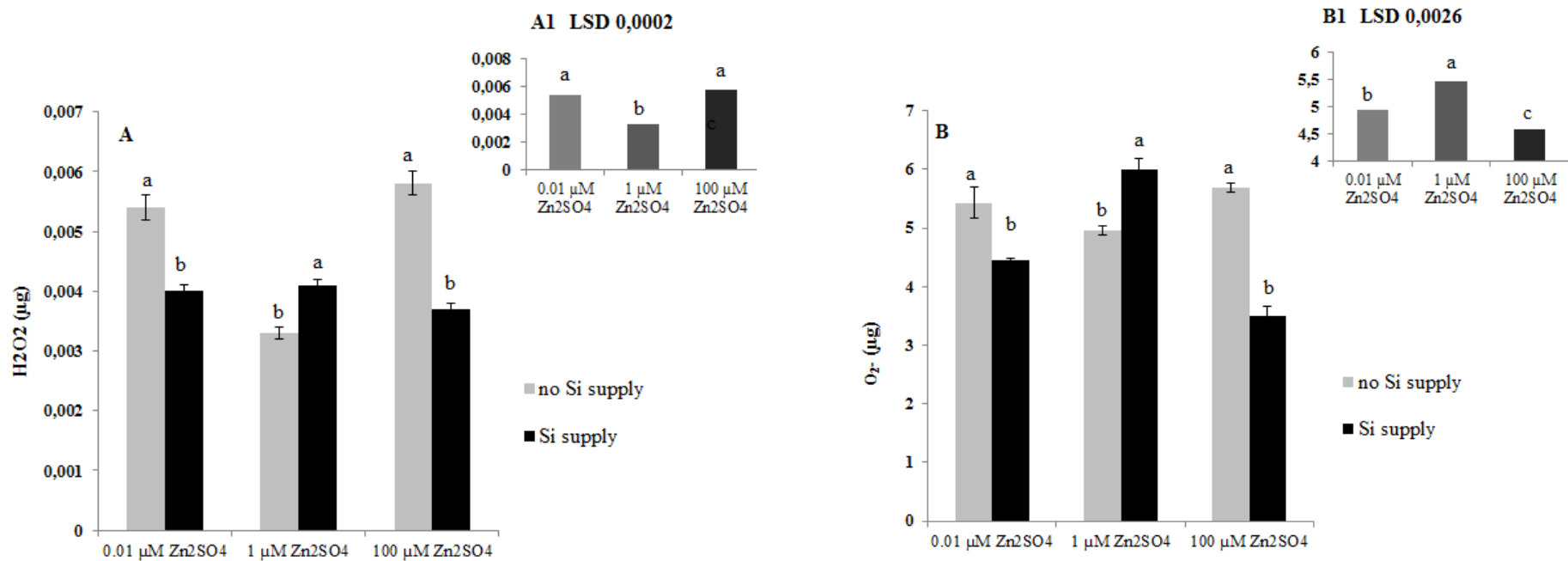


Fig. 15 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the concentration of H₂O₂ (µg) (A) and O₂⁻ (B) in barley leaves. The columns Values are mean ± standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05). A1 and B1. Values are means (control +Si supply n = 18) and differences between means were compared using LSD test (P =0.05). Levels of significance are represented by NS (non-significant) P > 0.05; * P < 0.05; ** P < 0.01 and *** P < 0.001 relative to the control.

4.10 Tricarboxylic acid cycle (TCA) enzymes

The study of the behavior of TCA enzymes in leaves of barley plants showed a similar response for citrate synthetase (CS) and fumarase (FUM) activity and an opposite response for phosphoenolpyruvate carboxylase (PEPCase) and malate dehydrogenase (MDH).

CS activity decreased in leaves exposed to Zn deficiency respect to the control (-41%); a similar but lower response was observed in Zn toxic plants (-16%). Si supply to the plant induced an increment of 100% in plants grown under Zn limitation and toxicity, reaching the highest level in the last treatment, while in plants grown with Zn control concentration, the silicate determined a reduction of 9% in enzyme activity.

FUM activity showed an appreciable diminution (-67%) in barley leaves treated with Zn deficiency, and a slight but significant decrease (-5%) under Zn toxicity. The application of Si determined different response in plants exposed to different Zn regimes. Respect to plant grown without Si supply, an increase of 67% was observed under Zn deficiency and a decrease of 50% in plants exposed to control concentration of Zn. No significant variation was recorded under Zn toxicity.

MDH activity rose its values when barley plants were exposed to Zn deficiency, doubling the control levels; similarly, an increase was observed in plants under Zn toxicity but with a lower rate (+38%). The combined treatment of different Zn nutrition and Si induced different responses depending on the Zn concentration. In Zn deficient plants determined an increase of 34% and it was unaffected in Zn control and toxic plants. PEPCase activity increased by 30% and 50% in Zn deficient and toxic plants, respectively. Si supply did not produce any change when plants were grown under optimal concentration of Zn but increased PEPCase activity in plants grown with low and high Zn concentrations (Fig. 16).

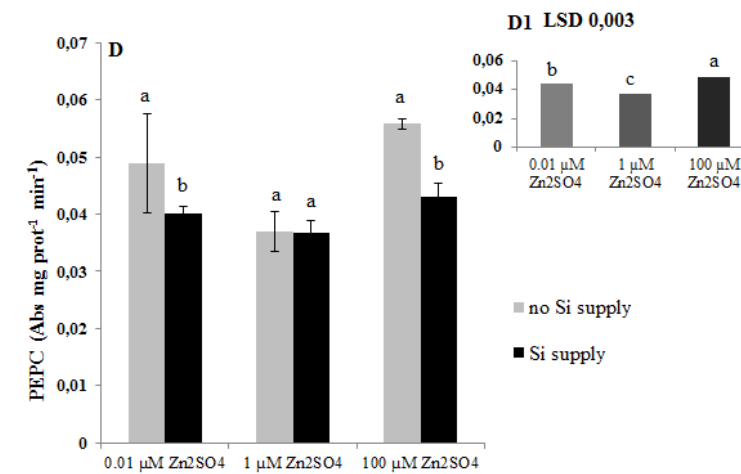
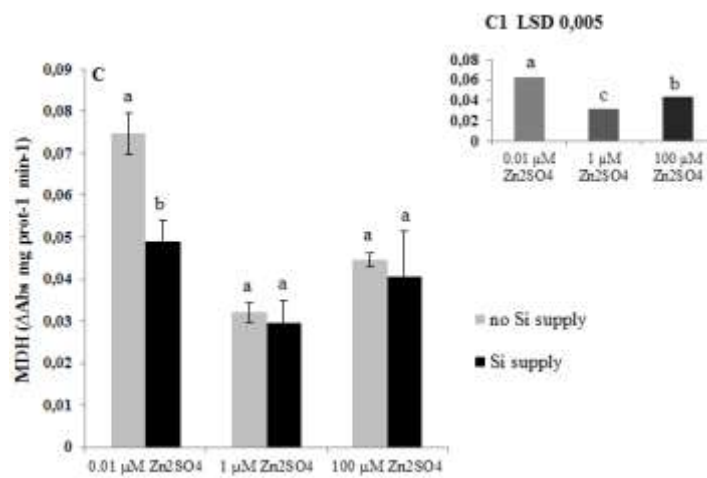
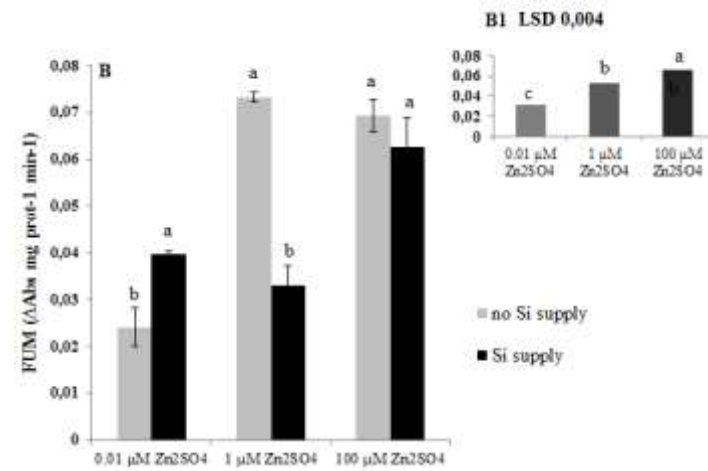
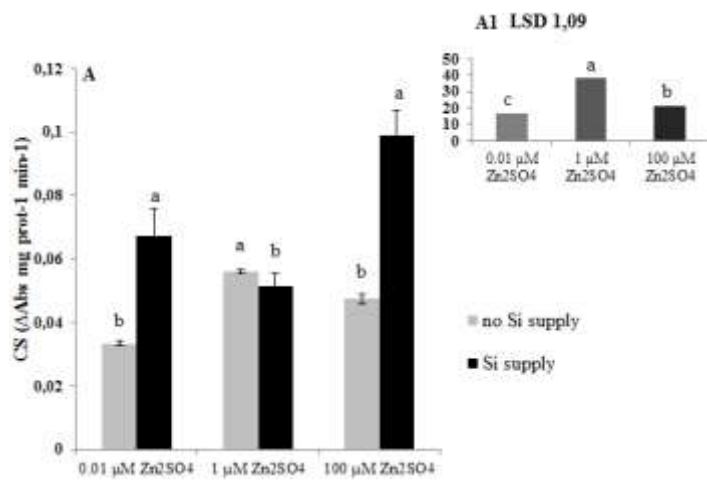


Fig. 16 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the activity of CS (Δ Abs mg prot⁻¹ min⁻¹) (A), FUM (Δ Abs mg prot⁻¹ min⁻¹) (B), MDH (Δ Abs mg prot⁻¹ min⁻¹) (C) and PEPCase (Δ Abs mg prot⁻¹ min⁻¹) (D) in barley leaves.

The columns Values are mean \pm standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05). A1, B1, C1 and D1 Values are means (control +Si supply n = 18) and differences between means were compared using LSD test (P =0.05). Levels of significance are represented by NS (non-significant) P > 0.05; * P < 0.05; ** P < 0.01 and *** P < 0.001 relative to the control.

4.11 Organic acid

The results showed that among all the OAs analysed, malate was the most important in the shoot of both species followed by citrate and oxalate as the least concentrated. Nevertheless, malate concentration did not change in barley plants under Zn deficiency, contrasting with the increment in citrate and oxalate concentrations. In plants grown under Zn toxicity it was recorded an increment in the levels of the three OAs studied. Si addition to the plant nutrition induced different responses depending on the OA considered. On citrate content, Si induced a decrease in plants grown under control and Zn toxicity, while no variations were registered in Zn deficient plants. Differently, Si supply increased malate content under control and Zn deficiency but not affected any way Zn toxic plants. Finally, considering Si application, oxalate content increased under both Zn deficiency and toxicity and not changed under Zn control plants (Table 15).

	Citrate ($\mu\text{mol/ g FW}$)			Malate ($\mu\text{mol/ g FW}$)			Oxalate ($\mu\text{mol/ g FW}$)		
	0.01 $\mu\text{M Zn}$	1 $\mu\text{M Zn}$	100 $\mu\text{M Zn}$	0.01 $\mu\text{M Zn}$	1 $\mu\text{M Zn}$	100 $\mu\text{M Zn}$	0.01 $\mu\text{M Zn}$	1 $\mu\text{M Zn}$	100 $\mu\text{M Zn}$
0 mM Si	4.69 \pm 0.01 ^a	4.17 \pm 0.01 ^a	5.09 \pm 0.06 ^a	8.87 \pm 0.18 ^b	9.04 \pm 0.05 ^b	10.78 \pm 0.07 ^a	0.10 \pm 0,01 ^b	0.08 \pm 0.01 ^a	0.10 \pm 0.01 ^b
1 mM Si	4.66 \pm 0.01 ^a	3.56 \pm 0.04 ^b	4.24 \pm 0.01 ^b	10.14 \pm 0.05 ^a	9.63 \pm 0.15 ^a	10.33 \pm 0.13 ^a	0.16 \pm 0.01 ^a	0.05 \pm 0.01 ^b	0.13 \pm 0.01 ^a
P-valor	NS	***	***	***	*	NS	***	**	*
0.01 $\mu\text{M Zn}$		4.69 ^a			9.51 ^b			0.13 ^a	
1 $\mu\text{M Zn}$		4.17 ^b			9.33 ^b			0.06 ^b	
100 $\mu\text{M Zn}$		5.09 ^a			10.55 ^a			0.12 ^a	
P-value		***			***			***	
LSD_{0.05}		0.06			0.21			0.01	

Table 15 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the citrate content ($\mu\text{mol/ g FW}$), malate content ($\mu\text{mol/ g FW}$) and oxalate content ($\mu\text{mol/ g FW}$) in barley leaves. Values are means (n=9) and differences between means were compared by Fisher's least-significance test (LSD; P=0.05). Values with different letters indicate significant differences. The levels of significance were represented by $p>0.05$: NS (not significant), $p<0.05$ (*), $p<0.01$ (**) and $p/<0.001$ (***).

4.11 Proteolytic activity

Compared to control, plants exposed to deficient concentration of Zn significantly increased proteolytic activity by 100%. A similar response was observed in plants grown in condition of Zn toxicity. Adding Si, the proteolysis was severely reduced by 20% both in Zn deficient than Zn toxic plants. Interestingly, Si seems to maintain protein degradation at the same rate as control under Zn stress. Contrarily, in control plants Si induced higher levels of proteolysis producing an effect similar to that observed in Zn stressed plants (Fig. 17).

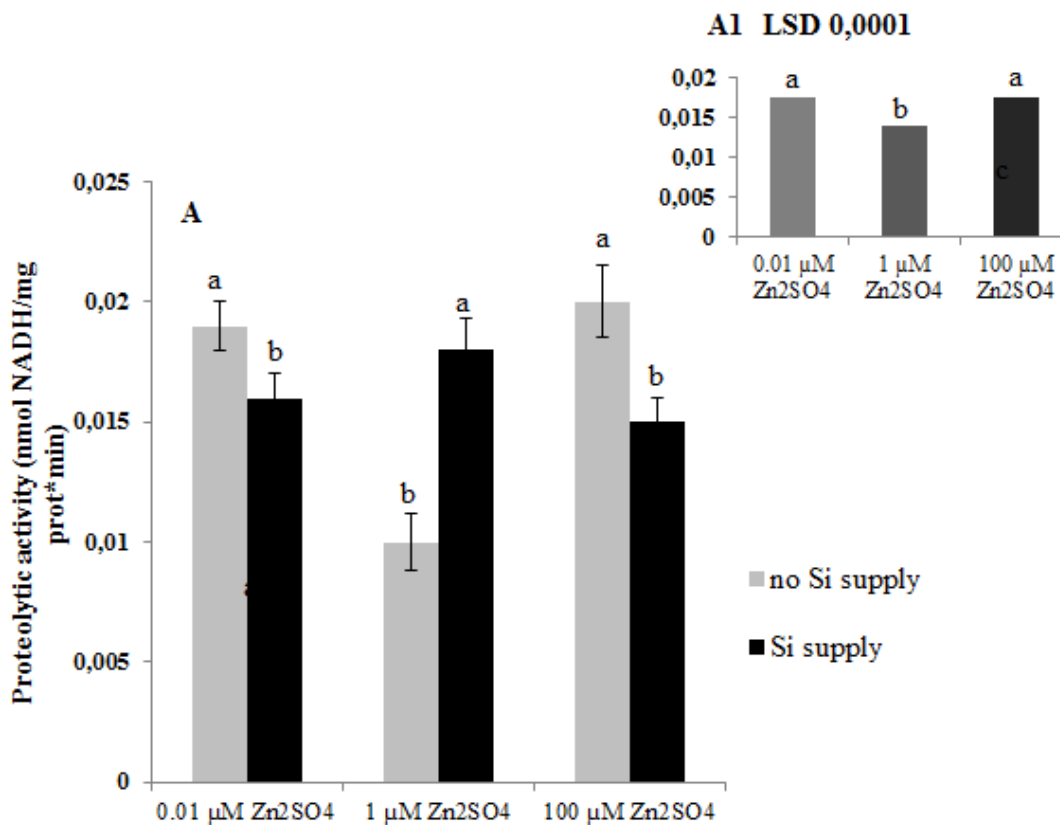


Fig. 17 Effects of different Zn nutrition regimes in the presence, or not, of 1 mM Silicon supply in the proteolytic activity (nmol NADH/mg prot*min) (A) in barley leaves.

Values are mean \pm standard error (n = 9), the differences between means were compared with the minimum significant difference of Fisher test (LSD; P= 0.05). A1 Values are means (control +Si supply n = 18) and differences between means were compared using LSD test (P =0.05). Levels of significance are represented by NS (non-significant) P > 0.05; * P < 0.05; ** P < 0.01 and *** P < 0.001 relative to the control.

5 DISCUSSION

5.1 Biomass and Zn concentration

Under stress conditions, plants tend to reduce growth (Wang et al., 2008b, Gong et al., 2013), mainly due to the fact that they need to invest energy in counteracting the stress to which they are subjected, or they are not able to obtain enough energy (Gong et al., 2014b). Biomass and relative growth rate are optimal indicators to evaluate plant stress and thus reflect plant growth (Gong et al., 2013).

Consequently, one of the most obvious symptoms of plants subjected to a Zn deficiency treatment is the decrease in biomass (Alloway 2008). Previous studies carried out in different species, showed a reduction in biomass in the presence of Zn deficiency (Ozturk et al., 2003, Wissuwa et al., 2006). Furthermore, in *L. sativa*, the most evident symptom of a deficit of Zn is the reduction in its biomass (Srivastava and Gupta 1996), as in *B. oleracea*, with biomass reductions of up to 62% in leaves in the presence of Zn deficiency (Hajiboland and Amirazad 2010b).

In this work, the results obtained show that the application of the deficiency treatment of Zn negatively affected the biomass of the barley seedlings, reducing 41% the dry leaf weight with respect to the control.

When exposed to an excess of Zn, plants accumulate this element in their tissues, where it can reach toxic concentrations, thus provoking metabolic alterations and inhibition of growth (Gisbert et al., 2006). Plants are not able to manage the excess of Zn by their physiological uptake systems, thus resulting in a serious threat to agricultural production (Vassilev et al., 2007). *L. sativa* reduced its biomass when exposed to toxic concentration of Zn (Paradisone et al., 2015), the same result has been observed in our experiments: barley plants subjected to 100 μM ZnSO_4 showed a decrease of 36% in leaf biomass.

Zn deficiency treatment induced a reduction of RGR by 20% and in Zn toxicity treatment by 30%. These results resemble and justify those previously described for biomass reduction.

Possibly, the decline in biomass and RGR under Zn stress, are related to both Zn accumulation and Zn deficit, as this micronutrient alter the basic physiological processes for the plants (Hafeez et al., 2013).

In our experiment, the Zn deficiency treatment resulted in a lower accumulation of Zn respect to control (a diminution of 40% Zn concentration was recorded). Previous works (Navarro-Leon et al., 2016), have also reported a lower accumulation of Zn during Zn deficiency treatments in both *L. sativa* and *B. oleracea* plants.

The Zn supplementation obviously influences the accumulation of this element in plant tissues: increasing the application of Zn in the medium, an increased accumulation of this element both in the aerial part and root (Zeng et al., 2011) was observed; this increase can be over 80% (Ozdener and Aydin ., 2010), due to possible compartmentalization mechanisms (Ma et al., 2005; Wojcik et al., 2005; Gisbert et al., 2006; Ozdener et al. ., 2010) or its precipitation in the apoplast (Küpper et al., 2000).

This agrees with our results in which Zn toxicity levels induced a 5.4 fold increase of Zn concentration in barley leaves.

The addition of Si to the growth medium, induced a levelling of leaf biomass to a value of about 80% of the levels observed under optimal conditions without Si; similarly, the leaf relative growth rate (RGR) under Si supply was about 90% of the maximum rate observed in controls. These data could be interpreted as a positive effect of Si, which is able to strongly reduce the growth limitation induced by Zn shortage or excess.

This could be the first step to alleviate plant stress due to not-optimal Zn concentrations. Under Zn control condition, the addition of Si did not change significantly Zn leaf concentration but induced a decrease in biomass and RGR showing that Si negatively affected plants grown with optimal Zn nutrition.

5.2 N metabolism

Abiotic stresses affect nitrogen (N) uptake and assimilation in plants. N is a primary plant nutrient that plays a crucial role in determining plant growth and productivity. Plants require nitrogen for the synthesis of vital molecules, such as proteins, nucleic acids and chlorophyll (Goel and Kumar Singh 2015). Zn deficiency in plants decreases yield and quality and result in sub-optimal nutrient use efficiency (Das and Green, 2013). In addition, it is known that an improvement of nitrogen use efficiency (NUE) is a major goal for crop breeders: selecting and growing N efficient crops represent a very special issue (Mifflin and Habash 2002; Van Bueren et al. 2014).

However, experimental data are still lacking to define specific micronutrients effects on the uptake and utilization of other essential plant nutrients (Fageria 2001). Likewise, the N-nutritional status of plants is an important critical player in root uptake and accumulation of Zn in plants (Erenoglu et al., 2011). The possible involvement of Zn in NUE, and consequently the concentration of total reduced N, suggests the interaction between this micronutrient and N metabolism, and therefore of the potential repercussions for plant growth and development. Our data showed that the value for reduced N was lower when the Zn doses applied were un-optimal. Smirnnoff and Stewart (1987) demonstrated that Zn deficiency caused a decrease in the concentration of reduced N compounds and shoot development. Additionally, the leaf N concentration not only represents N uptake and nutrition status in plant but is also highly correlated with plant growth and biomass (Kusano et al., 2011). Moreover, total nitrogen content (TNC) was also determined as indexes of the NO_3^- nutrition status of pure hydroponic grown (Hernandez et al., 1995). A similar pattern of this index with respect reduced N concentration was shown in our data.

The uptake of nitrogen, its translocation from root to shoot and finally its assimilation, has been found to be affected by high salinity in cowpea (Silveira et al., 1999).

The uptake of nitrate and ammonium is an active process mediated by nitrate transporters (NRT) and ammonium transporters (AMT), respectively. Harper and Paulsen (1969) found that NO_3^-

concentration could decrease in Zn deficient plants because these absorb less nitrogen than control plants. This could be caused by the impairment in cell membrane permeability and, therefore, the impairment in NO_3^- absorption (Welch et al., 1982). Goel and Kumar Singh (2015) suggested the downregulation of key genes involved in nitrogen uptake and assimilation in *Brassica juncea* L. exposed to abiotic stresses. Accordingly, *H. vulgare* showed a diminution of nitrogen uptake efficiency when exposed to low or high Zn concentrations, so indicating a possible downregulation of these genes in Zn-stressed plants.

Other index used for NUE definition is NUtE (Siddiqi and Glass 1981). In this respect our data show as a decrement or increment of Zn doses rise an increase of this parameter. These data suggest that barley plants could balance the decrease in N uptake with an increment of N utilization when exposed to Zn deficiency or excess. This could be the first response in nitrogen metabolism to alleviate Zn stress.

NO_3^- is the main N source for plants in most agricultural soils. It is absorbed by roots and transported to leaves, where it gives rise to assimilation products as amino acids (AAs) and proteins, needed for biomass production (Sivasankar and Oaks 1996).

It is known that Zn is required as structural and catalytic components enzymes for normal growth and development (Broadley et al., 2007). In fact, Zn is very closely involved in the N metabolism of plant (Mengel and Kirkby 1987). Respect to this metabolism, the enzyme NR plays a key role in the reduction of NO_3^- to NH_4^+ (Tischner 2000). Some studies have demonstrated a high inhibition of NR under Zn deficiency conditions in plants of *Oryza sativa* and *Pennisetum americanum* (Seethambaram and Das 1986) such as under Zn toxicity in: *Silene cucubalus*, *Deschampsia cespitosa* and *Triticum aestivum* (Mathys 1975; Smirnnoff and Stewart 1987; Luna et al., 2000). In our work, NR in stressed plants showed no significant differences from control plants: possibly this enzyme could be not affected by both low and high levels of Zn.

Several works show how in Zn limitation or excess plants, an accumulation of NO_3^- may occur due to the decreasing rate of reduction to NO_2^- (Harper and Paulsen 1969; Seethambaram and Das 1986).

On the other hand, despite of the stability of NR activity and the lower uptake in Zn stressed barley plants, NO_3^- content increased under metal stress. A possible hypothesis could be the diversion of reductants in the leaf from basal metabolism to stress response.

NH_4^+ concentration showed a significant increase in stressed plants. These levels are in accord with the possible increase in photorespiration that will be discussed after. It is well known that this process also produces significant amounts of NH_4^+ . Therefore, in our experiment, in Zn deficiency and toxicity plants, rate levels of NH_4^+ should be a consequence of increased photorespiration.

In most of higher plants, the primary assimilation of NH_4^+ into glutamine (Gln) is through the cooperative activity of GS and GOGAT (Igarashi et al., 2006). In fact, since NH_4^+ is highly toxic in plants, this is quickly absorbed by the GS/GOGAT cycle. In this respect, GS plays a central role in the N metabolism and has been considered as the mayor assimilatory enzyme for ammonia produced from N fixation, and NO_3^- and NH_4^+ nutrition (Miflin and Habash 2002).

Our results show that NH_4^+ concentration in the leaves partly increased under not-optimal Zn levels. Additionally, GS activity was strongly and positively affected by Zn doses different from optimal. According to our results, Thomsem et al., (2014), have demonstrated a strongly correlation between the levels of NH_4^+ and the GS activity. These authors concluded that an overexpression of GS in leaves could be advantageous for the efficient NH_4^+ assimilation and the export of amino acids via phloem promoting the plant growth.

Glutamate dehydrogenase (GDH) can be involved in NH_4^+ assimilation when reduced N is highly available and/or concentrated in plant tissues (Robinson et al., 2014). GDH catalyzes the reversible reaction of NH_4^+ and 2-oxoglutarate to glutamate (Glu). However, this double physiological function has recently stimulated a considerable debate, because GDH has been found to have a high

$K_{m_{NH_4^+}}$ indicating that it is improbable that it would act in favor amino acid biosynthesis. In fact, in barley leaves the activity of GDH increased when Zn doses were different than control. Some studies have revealed that GDH levels also increase under various stress conditions and again the plant may well need to give priority to carbon metabolism and keto-acid production over N metabolism (Mifflin and Habasch 2002). Frechilla et al. (2002) indicated that GDH also participates in the assimilation of N in the presence of GS when the concentration of NH_4^+ is elevated.

Finally, the enzyme AAT plays a key role in the metabolic regulation of C and N metabolism in all organisms (Torre et al., 2014). In general, but particularly under C shortage, the operation of AAT will ensure that the different keto-acids needed by the plant will be available (Mifflin and Habasch 2002). In this respect, *H. vulgare* showed a similar pattern of the AAT activity respect to GS, that is, an increase of AAT activity in Zn stressed plants. An increase of GS could promote a higher use of Gln and Glu produced from the cycle GS/GOGAT and subsequently the formation of aspartate (Asp) and others essential amino acids required for development by the AAT activity. As mentioned above, the biomass of the aerial part of barley was affected negatively under Zn limitation and excess and this fact could be due to a greater amino acid synthesis by AAT activity but not so efficient to contrast the stress induced by the micronutrient.

The result of NH_4^+ assimilation can be quantified by analyzing the total reduced N, which is usually the product of assimilation of N and consists mainly of AAs and proteins. Therefore, it is an essential parameter to determine the plant nutritional status (Ruiz and Romero 1999). Our results show that total reduced N concentration is negatively affected by Zn stress in barley. This is in contrast with the higher GS/GOGAT activity which occurs as a result of Zn deficiency and toxicity. As observed for leaf growth parameters, nitrogen assimilation and metabolism looked ameliorated by Si supply: the general effect on NUpE values confirmed that Si strongly reduces the effects of Zn limitation or excess. Thus, this parameter is reported to values that are – although lower than controls without Si – acceptably higher to reduce stress effects. No Si effects were observed on

NutE parameter for Zn stress conditions. Differently, in Zn control condition, Si inhibited nitrogen uptake efficiency but improved the nitrogen utilization.

In Zn deficiency and toxicity, according with the NUpE and NUtE values, Si induced an increment of nitrogen uptake which was shown by higher levels in all the nitrogen forms (NO_3^- , N organic and TNC). In Zn deficient plants, Si increased NR activity since an increment of its substrate, NO_3^- , however, NH_4^+ did not increase probably due to the huge increment in GDH activity that probably is able to use ammonium to produce glutamate. Differently, in Zn toxic plants, Si inhibited NR activity, as supported by of NO_3^- increment; however, it was observed an increment of NH_4^+ levels probably induced by an increment of reversion GDH activity in the deamination side, producing ammonium and keto-glutaric acid that could enter into the Krebs cycle. It is interesting to observe that GS activities, that are sensibly increased under Zn limitation and, particularly, under Zn excess, were reduced to control values by Si supply.

These results can be interpreted with a reduction of ammonium recycling through photorespiratory cycle (as it will be discussed later) and therefore as a reduction of stress symptoms. Furthermore, a diversion of nitrogen assimilation from photorespiration to GDH reaction could be suggested by the strong increase of this enzymatic activity under supply. Under Zn control condition, despite the lower N uptake efficiency, we observed a higher N utilization efficiency that is confirmed by higher levels of NO_3^- and NH_4^+ concentrations and an enhancement in NR and GS activity.

5.3 Photorespiration

Stress conditions in plants have effect on both photosynthetic rate and nitrogen metabolism. To this aim we investigated both the main N accumulation and assimilation pathways, and photosynthesis/photorespiration.

Preliminarily, it should be observed that in addition to that produced from NO_3^- reduction, NH_4^+ is continually formed during various metabolic processes in the tissues of higher plants. These

processes are generally called secondary NH_4^+ assimilation and includes amino acids and proteins catabolism, phenylpropanoid biosynthesis, and, especially, photorespiration.

Photorespiration is essential in maintaining the adequate N level in the plant since it produces significant amounts of NH_4^+ which can be 20-fold more than the generated by NO_3^- reduction (Hirel and Lea, 2001; Wingler et al., 2000). At this regard, the photorespiration suppression has negative effects on plants, producing a decrease in the CO_2 assimilation rate and alterations in chloroplast structure. Shi-Wei et al. (2007) indicated that this process can provide protection against photoinhibition and increases protection against different types of stress. Likewise, the photorespiration appears to be an essential process in the control of amino acid biosynthesis and metabolism (Igarashi et al., 2006). Furthermore, the photorespiratory nitrogen cycle contributes to the metabolism of specific key amino acids as Glu, Asp, Ser and Gly. Several enzymes from different organelles are involved in photorespiration, including glyoxylate oxidase (GO) and glutamate: glyoxylate aminotransferase (GGAT) in peroxisomes. In our experiment Zn deficiency and toxicity caused an increase in photorespiratory cycle, as indicated by a large increase in GO and GGAT activities with respect to controls. This could be a reason for the resistance against Zn stress, since greater photorespiration activity can help in ROS elimination (Wingler et al., 2000). In a previous study on rice (Seethambaram et al., 1985) similar results were observed: Zn deficiency caused an increase in photorespiration, and hence a higher release of NH_4^+ by decarboxylation of Gly could compensate the failed increment in the NO_3^- reduction under stress.

Si seemed to inhibit photorespiration under Zn stress conditions decreasing GO and GGAT activities, even if any significant variations were observed in GGAT activity of Zn toxic plants. Conversely, under Zn control condition, Si application increased photorespiration rate.

5.4 N derived protective compounds

N derived protective compounds act as organic compatible solutes and normally they are not toxic at high concentrations in the cell (Serraj and Sinclair, 2002). These compounds are able to protect

plants against stress by adjusting the osmotic potential, detoxifying ROS, protecting membrane integrity and stabilizing enzymes and proteins (Bohnert and Jensen, 1996). Among these compounds are Pro and GB (Ashraf and Foolad, 2007). According to our results, Zn deficiency and toxicity causes an increase in Pro and GB concentration in *H. vulgare* plants compared to controls. This is consistent with what was observed in cabbage plants (*B. oleracea*) (Hajiboland and Amirazad, 2010) and rice plants (Höller et al., 2013) in which Pro concentration increase over control in Zn deficient plants. Paradisone et al., (2015) demonstrated that in *L. sativa* Proline levels increased under Zn toxicity as a symptom of stress. Accumulation of proline is an indication of protection for plants under environmental stresses (Chen et al., 2003). Increases in proline as a function of metal accumulation have been observed in response to heavy metals, including Zn (Bassi and Sharma 1993). Zn deficiency and excess stimulated accumulation of proline, which apart from acting as a metal chelator and osmolyte, scavenges hydroxyl radical (OH•) and ¹O₂ and thus provides protection against ROS induced cell damage (Radić et al., 2009) to some extent. The accumulation of Pro under Zn stress could be caused by a direct effect of Zn on Pro synthesis in these plants: it could be argued that a higher synthesis of Glu as precursor for proline synthesis is necessary, thus inducing an increase in GS/GOGAT and GDH activities.

Navarro-Leon et al. (2016) showed that Zn deficiency produced an increment of GB concentration in *Brassica rapa*. Similarly, our data showed an increase in the compound content when plants were exposed to sub-optimal Zn dose. Several studies reveal that GB accumulates in response to heavy metal stress in plants. Barley plants treated with 100 μM Zn showed higher levels of GB as previously reported upon abiotic stress (Cardi et al., 2015).

Considering the GB synthesis route in plants, if Ser accumulates it will enhance GB synthesis. Thus, according to our results, the higher GB concentration under Zn deficiency and toxicity could be explained by a stimulation of its synthesis due to the increment in Ser content: there is an increase in GO and GGAT activities so hydroxypyruvate accumulates and hence Ser would accumulate as well.

Since *H. vulgare* is sensitive to Zn stress, as confirmed by the significant loss of plant biomass, our data contrast with the hypothesis that GB accumulation represents a symptom of an enhanced tolerance to abiotic stress (Sakamoto and Murata 2000). Finally, as previously suggested, it is assumed that Pro and GB increment do not represent a signal of resistance to toxicity, but probably a symptom of Zn stress (Paradisone et al., 2015).

The positive effects of Si were accompanied by a reduction of proline levels. Therefore, it could be argued that proline levels acted as a stress sensor more than a stress effect. In contrast, glycine-betaine concentrations resulted substantially insensitive to Si supply.

5.5 Amino acids concentration

The amino acid metabolism is tightly linked to carbohydrate metabolism, NH_4^+ (absorbed and synthesized from NO_3^-), and demand for protein synthesis and secondary metabolism. For this reason, the regulation of amino acid content, fluxes and transport through the plant is thus critical for plant adaptation to carbon and nitrogen status, development and defense (Patrelli and Pilot 2014). In general, on the basis of our results we can confirm as a Zn deficiency or toxicity condition can promote an increase in the total amino acid concentration. The photorespiratory nitrogen cycle contributes to the metabolism of certain amino acids (Glu, Ser and Gly). In this way GGAT functions cooperatively with the other photorespiration enzymes in the production of amino acids. In barley plants, a GGAT increase can promote the integration of carbonated skeletons from photorespiration into the Calvin cycle, promoting the reduction and assimilation of N. Thus, low and high Zn doses could be sufficient for maintenance a suitable balance C/N promoting the amino acids synthesis. In particular, we could not recognize any amino acids that was significantly affected by Zn deficiency; on the other hand, high Zn doses induced a huge increment in two amino acids, aspartate and serine, probably as response to increasing photorespiration and oxidative stress. Si decreased levels of amino acids involved in the photorespiration. Moreover, it seems to decrease levels of Glu, Cys and Gly, amino acids needed for GSH synthesis. This could be interpreted as a

lower turnover of GSH molecules according to the reduced oxidative stress in presence of Si, since the ratio GSH/GSSG become more favorable (increment of total GSH). Arg levels diminished in plants treated with Si, probably for the greatest free NH_4^+ accumulation.

5.6 Photosynthetic parameters

Photosynthesis is a vital process in plants, it synthesizes organic components, provides energy and directly determines biomass production. This process is highly sensitive to any stress. In order to carry out photosynthesis, it is essential to absorb the light energy by chlorophyll (Pfannschmidt and Yang 2012). SPAD meters determine the greenness and the interaction of thylakoid chlorophyll with incident light, estimating the chlorophyll content. This technique allows making *in vivo* measurements since it is not destructive and is a good indicative of the damage caused in the photosynthetic system (Jifon, Syvertsen, and Whaley 2005). In the present study, SPAD values correlated well with plant phenotypes since plants submitted to low Zn and high Zn doses presented more chlorotic aspect and lower SPAD values, and the opposite for plants submitted to control Zn dose with higher SPAD values and a greener phenotype. It exists a strong positive correlation between SPAD index and Chl concentration (Kalaji et al., 2016). Thus, other experiments showed reductions in Chls concentrations under Zn deficiency and toxicity; for instance in pistachio (Tavallali 2017), maize (Wang and Jin 2005), peach (Song et al., 2015) and in cotton (Anwaar et al., 2015). Chls concentration may be reduced by a lower biosynthesis, changes in chloroplast ultrastructure and photo-deterioration (Xiao, Yang, and Lee 2016). Alternatively, it could be an adaptive mechanism to prevent photoinhibition by decreasing light capture (Choinski Jr, Ralph, and Eamus 2003). These effects appear to be mitigated by Si in our experiment. Thus, according SPAD results, Si application protected somehow barley plants against the chlorosis caused by Zn deficiency and toxicity in the plant. However, according Chls concentration Si only was effective in plants that received low and control Zn doses and not in the high Zn dose. Other authors also observed this protector effect of Si on pigments. For instance Si application increased SPAD levels

in rice (Ranganathan et al., 2006; Kim et al., 2011) and in mung bean Si application increased Chls concentration (Ahmad et al., 2018).

Chl a is mainly associated with reaction centres and core antenna proteins of PSII while Chl b is mainly present in light-harvesting complexes (LHCII). Therefore Chl a/b ratio is a good indicator of RC/ LHCII proportion, when this ratio declines is a symptom of stress response in plants and it often is linked to a reduction of photosynthetic capacity (Nyongesah, Wang, and Li 2015). When Chl a/b ratio is higher, it could indicate a conversion of Chl b to Chl a, in order to maintain Chl a levels and, thereby active RCs (Yang, Kong, and Xiang 2009). This could be favoured by Si application under Zn toxicity, since plants presented a higher Chl a/b ratio than without Si and it suggest a role of Zn homeostasis in Chl a/b ratio adjustment. Nevertheless, in the present work, the different Zn doses did not affect the Chl a/Chl b ratio. Regarding Chl a/Chl b ratio, other experiments showed that Zn deficiency increased Chl a/Chl b ratio due to Chl b was more negatively affected than Chl a under Zn deficiency (Wang, 2005; Tavallali, 2017). In addition Si also increased Chl a/Chl b ratio in maize (Sacała 2017) and wheat (Ali et al., 2016).

Carotenoids also are components of thylakoids in chloroplasts and play a role as accessory light-harvesting pigments and as antioxidant compounds eliminating ROS (Havaux 1998). The results for carotenoids suggest that Zn deficiency promote the accumulation of these pigments, probably in order to respond to Zn deficiency stress. However, under Zn toxicity this response appears to be inhibited as carotenoids levels were not affected. In contrast in another study carried on red cabbage Zn deficiency did not affected to carotenoids concentration in red cabbage (Hajiboland and Amirazad 2010) and in cotton, Zn toxicity decreased carotenoids accumulation, so the response of carotenoids to Zn appear to be specific on the species. On the other hand, in our experiment Si application could be positive to increase carotenoids content when control and high Zn doses are applied. This result agrees with which observed in other studies. For example, in cotton, Zn toxicity decreased carotenoids accumulation, however Si application increased it (Anwaar, 2015) and also in mung bean when NaCl stress was applied (Ahmad, 2018). Therefore, Si application could be useful

to increase carotenoids content in plants. This could be important to improve antioxidant proprieties in food crops for human diets (Nicolle et al., 2004).

It has been proved that Chl a fluorescence well reflects the state of plant photosynthesis and the changes produced on this due to a stress. When plant metabolism is disturbed, fluorescence is usually produced to dissipate redundant energy in order to avoid damage (R. Strasser, Srivastava, and Tsimilli-Michael 2000). All dark-adapted plants analysed in other works presented a characteristic variation with time in its Chl fluorescence. Fluorescence describes a polyphasic curve with several steps (O, J, I, P): O, first measurement at the beginning of illumination, J at about 2 ms, I at about 30 ms and P at about 500 ms. The fluorescence transient can be quantified using the JIP test that provides parameters indicating the in vivo PS II performance (Strasser *et al.*, 1999; Strasser *et al.*, 2000). A parameter derived from JIP test is initial fluorescence at O (F_o). After the dark adaptation period, all the primary quinone acceptors in PSII are in the oxidized state, so a high F_o indicates a lower proportion of these acceptors available for reduction as result of a stress. In addition, higher F_o could indicate a chronic loss of active RCs (Tsimilli-Michael and Strasser 2008). The different applications of Zn did not affect to this parameter so is not probable a the loss of active RCs. This agree with was it observed in a study employing several plant species grown under Zn deficiency where F_o values did not change (Zhao and Wu 2017).

Fluorescence value at P is named F_m and expresses the fluorescence when all the RCs are closed. Variable fluorescence (F_v) is the difference between F_m and F_o and indicates the maximum capacity of photochemical quenching (R. Strasser, Srivastava, and Tsimilli-Michael 2000). Based on F_v and F_m values JIP test provides the quantum yield of primary photosynthesis (F_v/F_m) that is good indicator of plant photosynthetic performance and is broadly used. F_v/F_m indicates the maximal energy flux which reaches to the PS II reaction centres (R. Strasser, Srivastava, and Tsimilli-Michael 2000). Our results showed that Si could enhance this energy flux to the PSII under conditions of Zn stress since plants on these treatments presented higher F_v/F_m values. Based on measurement in several plant species it was observed that F_v/F_m of healthy leaves often reach

values close to 0.83 and values below usually indicates the damage of PSII reaction centre and a reduced electron transport capacity (Guidi and Degl'Innocenti 2012). Thus, we observe the lowest values under Zn toxicity and in the rest of the treatments Fv/Fm values are close to 0.83 so these treatments do not affect dramatically to PSII performance. In other experiments it is observed that when Zn supply is unadequate Fv/Fm decrease in maize by Zn deficiency (Wang, 2005) (Mattiello et al., 2015) and by Zn toxicity in black oat (Tiecher et al., 2016) or not affected in red cabbage by Zn deficiency (Hajiboland, 2010). Anyway, Si is able to increase Fv/Fm for instance in tomato seedling (Sivanesan et al., 2011).

The photosynthetic performance index (PI_{ABS}) is an indicator of plant vitality and shows the plant capability to resist external pressures. PI_{ABS} is a combined index that depends on three factors: the concentration of active RCs (RC/ABS), the above mentioned Fv/Fm, and the efficiency which electrons move further than Q_A^- (Ψ_o) (R. Strasser, Srivastava, and Tsimilli-Michael 2000). The proportion of active RCs determines how much energy is emitted as fluorescence and how much is usable for photosynthesis and under stress RCs can be transformed in quenching sinks that not reduce Q_A to Q_A^- , limiting photosynthesis yield (Strasser *et al.*, 1999). Quantum yield of electron transport (Φ_{Eo}), express the probability that an absorbed photon moves an electron through PSII (R. Strasser, Srivastava, and Tsimilli-Michael 2000). We observed that Si increased PI_{ABS} in all the analysed plants. This increment is because of Si improved all of the three components of PI_{ABS} . Therefore, this is a good indicative that Si improves photosynthetic performance. In barley plants grown under high Zn supply, the reduction of Ψ_o and Φ_{Eo} could lead to a higher accumulation of reduced Q_A^- due to the minor electron transport activity (Tan et al., 2011).

The time to reach Fm (tFm) and the number that QA- is reduced form time 0 to the time to reach tFm (N) are also good indicators of photosynthesis functioning since their values are usually lower when plants are subjected to stress. The higher tFm and N parameters, the more Q_A^- can be potentially reduced (R. J. Strasser, Tsimilli-Michael, and Srivastava 2004). According to the results, high and especially low Zn dose reduce the quantity of Q_A^- that can be reduced which could limit

the photosynthesis rate. However Si increased tFm and N which could be due to an improved photosynthetic efficiency.

On the other hand, fluorescence at 0.3 ms (K step) usually increases under stress and indicates instability of the oxygen evolving complex (OEC) and it produces an uncoupling between OEC and the rest of PSII (R. Strasser, Srivastava, and Tsimilli-Michael 2000). Barley plants that received Si treatment showed reduced K step values disregarding the Zn application, which indicate a better electron transference between OEC and the rest of PSII components.

The area above the fluorescence curve between Fo and Fm is proportional to the size of the pool of Q_A^- in the PSII. Additionally, Sm parameter is the measure of the energy necessary to close all the RCs through the Q_A^- reduction and the next electron acceptors. Usually, if the electron transfer from the RC is blocked, Area and Sm values are reduced (R. Strasser, Srivastava, and Tsimilli-Michael 2000). Thus, we observed this reduction when we subject barley plants to Zn deficiency and to Zn toxicity. The shortage and the excess of Zn affect to electron transference from RC to electron acceptors. However, Si application contributes to protect against this, allowing a better electron flux.

Furthermore, the analysis of Chl a fluorescence is able to inform us which part of PSII is affected by stress through the analysis of electron fluxes. Stress can damage flux at pigment antenna level (ABS/RC), at RCs (TR/RC), or after Q_A^- reduction (ET/RC) (R. Strasser, Srivastava, and Tsimilli-Michael 2000). Our results show that both Zn deficiency and toxicity affected electron flux because these treatments reduce the values of fluxes parameters. However, we noted that Zn deficiency affect in a more severe way since it reduced ABS/RC and TR/RC in a greater proportion. Our results agree with those observed by Zhao (2017) since the Electron Transport Rate (ETR) was reduced in all species subjected to Zn deficiency. On the other hand, Si application to the nutritive solution was able to increase all the fluxes but only in plant supplied with control and high Zn doses. Si appear to be unhelpful to increase ABS/RC and TR/RC fluxes under deficiency conditions as these parameters decreased slightly with respect control conditions.

In summary, considering all the results for Chl fluorescence, we can conclude that both Zn deficiency and toxicity affect to barley plant photosynthesis. Zn deficiency affected in greater extent than Zn toxicity which is reflected in decreases in tF_m , N and ABS/RC and TR/RC fluxes. Likewise, Zn misbalance affected negatively especially to electron in antenna pigments indicated by lower ABS/RC and flux after Q_A^- as indicated by lower Ψ_0 and ET/RC . This produces an increase in energy dissipation in the form of fluorescence as indicated by lower $Area$ and S_m values. On the other hand Si application reduced this loss of energy as fluorescence and enhanced photosynthesis performance especially under Zn stress as indicated by an improvement in the majority of parameters.

5.7 Oxidative stress

Zn ions stabilize and protect cell membranes and enzyme proteins against oxidative attack of toxic O_2 species by binding sulfhydryl groups and phospholipids (Willson, 1989; Bray and Bettger, 1990). Toxic O_2 species such as the superoxide radical (O_2^-) and hydrogen peroxide (H_2O_2) are produced in high amounts in various cell compartments, especially chloroplasts of higher plants (Elstner, 1982). Thus, Zn ions also participate in enzymatic defense of cells against free radical damage (Cakmak and Marschner, 1993). An excess in ROS production causes cell damage, affects DNA, oxidizes AAs and proteins and results in lipid peroxidation (Karuppanapandian et al. 2011). There are numerous oxidative-stress indicators in plants but we focused our attention on O_2^- and H_2O_2 since they are known to be determinants in stress process.

In the present work, plants subjected to sub-optimal concentration of Zn, accumulate O_2^- , showing a high oxidative stress. This result was in accord with Cakmak and Marschner (1988), who described an enhanced superoxide radical production in roots of Zn deficient cotton plants. The behavior observed in plants exposed to Zn deficiency was recorded in plants exposed to Zn toxicity too. Supra-optimal levels of Zn stimulate ROS production inducing an oxidative stress (Lin and Aarts,

2012). For this reason, the activation of detoxification mechanisms plays a key role in alleviating toxic Zn effects (Wang et al., 2009).

Gururani et al. (2015) observed that ROS adversely affect the photosynthetic system through the inhibition of chloroplast enzymes, the disruption of thylakoid structures, and the hindering of PSII repair process. The chloroplast is an important source of ROS due to the photosynthetic O_2 synthesis and the high energetic electron transport, that – if not adequately regulated – resulted in ROS formation. When electron transport chain is damaged, elevated excitation pressure leads to ROS production and these in turn produce more damage. Indeed, total Chl reduction can be an indicator of damage caused by ROS (Foyer et al., 1994).

Accordingly, the high oxidative stress observed in barley plants could be responsible for the altered photosynthetic performance observed in this study. To prevent the oxidative damage, in chloroplast there are different antioxidant compounds such as carotenoids that prevent and eliminate ROS accumulation (Havaux 1998). Thus, in our study, the results for O_2^- (Fig.), total Chls and carotenoids (Table) suggested that barley plants exposed to not-optimal Zn levels presented the higher oxidative stress and lower antioxidant capacity under low and control conditions.

Dismutation of O_2^- by SOD or non-enzymatic reactions produce H_2O_2 which is also toxic and essential for the production of the powerful oxidant hydroxyl radical, OH.

In this work, despite the O_2^- accumulation and the probable SOD activity decrease, plants exposed to Zn stress recorded a significant increment of H_2O_2 levels.

Plants have mechanisms to eliminate ROS through antioxidant enzymes such as APX, GR and CAT activity that are efficient H_2O_2 scavenger (Du et al., 2001).

The current literature shows how APX activity could have different response under Zn deficiency and toxicity depending on the species. Cakmak and Marschner (1993) demonstrated that APX and GR were severely decreased in bean leaves grown under Zn deficiency. Barrameda-Medina et al. (2014) found no significant influence in *L.sativa* exposed to Zn toxic concentrations; accordingly, in our experiment different Zn nutrition did not influence APX activity. This stability may be due to

the no effects of Zn concentrations on this enzymatic activity. The enzyme APX is thought to play the most essential role in scavenging ROS and protecting cells in higher plants because its high affinity for H₂O₂ (Gill and Tuteja 2010). Thus the unincreased activity of peroxidase could explain the high H₂O₂ accumulation in these plants.

However, the H₂O₂ detoxification could be carried out by other ways such as the GR and CAT enzymes.

In addition to APX, GR is another efficient H₂O₂ scavenging system of particular importance present in chloroplasts (Asada 1992). APX detoxifies H₂O₂ and GSSG reductase is involved in regeneration of AsA as well as of NADP⁺; the latter accepts electrons from ferredoxin and thus limits photoreduction of O₂ to O₂⁻. In many instances, resistance against photooxidative damage to thylakoid constituents is correlated with elevated levels of this antioxidative enzyme (Bowler et al., 1992; Gressel and Galun, 1993).

Our data showed that GR activity was increased at both deficient and excess level of Zn supply as previously described (Lascano et al., 1998; Pandey et al., 2005; Gupta et al., 2011).

In contrast, Cakmak and Marschner (1993) observed a low level of GR activity under Zn deficiency. Increased levels of GR in response to Zn stress is possibly an adaptive feature contributing to tolerance against stressful conditions by sustaining the pool of reduced glutathione at a high level of Zn.

Prasad et al. (1999) observed an up-regulation of CAT activity in *Brassica juncea* grown under conditions of excess Zn. Similarly, Tewari et al. (2008), studying the effects of Zn deficiency and toxicity on mulberry plants, demonstrated the intensification of CAT activity under Zn stress. Accordingly, our results showed that un-optimal Zn concentration positively affect catalase activity. For a complete assessment of the antioxidant response, an analysis was made of the concentrations of non-enzymatic antioxidant systems. The levels of non-enzymatic antioxidants (AsA and GSH) are indicators of the redox state of the plant and responsible in part for resistance to stress. The cycle ascorbate-glutathione represents a complex antioxidant system. In the ascorbate-glutathione

cycle, the APX enzyme reduces H_2O_2 into water using ascorbate as the electron donor and the resulting dehydroascorbate (DHA) is cycled back to ascorbate using reduced GSH as the electron donor, while the GSSG formed is converted back to reduced GSH by NAD(P)H-dependent glutathione reductase (Gomes et al., 2013). The presence of cycle AsA-GSH in all the cellular compartments and the high affinity of APX activity for H_2O_2 suggests that this cycle plays a crucial role in ROS equilibrium (Mittler 2002; Seth et al., 2012).

Subba et al. (2014) affirmed that leaf tissues of the mandarin subjected to Zn-deficiency and excess, generated AsA at lower rate. Tsuji et al. (2003) demonstrated that in *Dunaliella tertiolecta* under Zn stress, GSH was increased by the induction of the enzymatic synthesis of this compound. Similarly, in Barrameda-Medina et al. (2014) the response of both antioxidants to Zn excess was a major increase in the oxidized forms of AsA and GSH. These data are confirmed by Cuypers et al. (2001) indicating that the cellular redox status of AsA and GSH pools responds strongly to Zn phytotoxicity. In our work, both Zn deficient and excess plants presented a decrease in the redox status of the two antioxidants with a decrease in their ratio reduced/oxidized forms. These data confirmed an intensification of the ascorbate-glutathione cycle, as previously hypothesized, given the increase of GR activity and the not inhibition of APX activity under Zn stress.

ROS data confirmed the beneficial effect of Si on stress tolerance: O_2^- and H_2O_2 decreased their levels respecting to treatments without Si. These decrements could be correlated with the increment on plant biomass and RGR. Furthermore, the lower ROS levels in plants treated with Si could be caused of a better photosynthesis performance (as showed above), that could reduce ROS generation. These effects were not observed under control condition of Zn. The variations on CAT activity could be due to the different concentrations of its substrate, H_2O_2 , with Si supply: CAT activity decreased in Zn deficient plants contrarily to an increment of the activity in Zn control condition. This correlation was not observed in Zn toxic plants, where a decrease of H_2O_2 was not correlated with a decrease of CAT activity that stayed stable to the level of treatment without Si. Similarly, APX activity decreased with Si supply in all the treatments studied: Si inhibited the APX

activity in the Zn stressed plants, decreasing the substrate levels (H_2O_2) while in control plants the reduced activity is due to the higher H_2O_2 detoxification through CAT activity. Consequently, the lower APX activity was correlated with a lower DHA concentration in Zn stressed plants, while no Si effects were registered in control plants. In Zn limited plants treated with Si, the higher AsA reduced concentration could be positive to face Zn deficiency. However, Si supply was not effective to increase AsA level in Zn excess condition. The not beneficial Si effects were again confirmed by a decrease of the reduced ascorbate in Zn control plants. Similar response was observed in glutathione concentration: Si induced in Zn stressed plants a decrease in oxidized form and an increase in reduced form. The decrease in GSSG form could be the cause of the reduction in GR activity, thus the GSH accumulation could be caused by an inhibition of DHA reductase.

These data were confirmed by the redox state of ascorbate and glutathione that are shown to be lower in stressed plants (Cuypers et al., 2001). Si enhanced redox state of ascorbate and glutathione and this could be positive to face Zn stress in barley plants.

5.8 Carboxylate metabolism

Few authors investigated the effects of micronutrient deficiencies on TCA enzyme activities. The most of published works prove that the responses depends on the species and the micronutrient stress.

PEPC activity was enhanced by Fe deficiency in tolerant genotypes of *Pisum sativum* (Jelali et al., 2010) and in *Beta vulgaris* (López-Millán et al., 2001) while it was not affected by Zn toxicity in *L. sativa* and *B. oleracea* plants (Barrameda-Medina et al., 2014). In the present work Zn deficiency and toxicity stimulated PEPC activities in barley plants suggesting that this could be a strategy to synthesize more citrate and oxalate in order to sequester Zn under deficiency conditions.

FUM and MDH activities showed mixed results under micronutrient deficiencies in previous works. These two enzymes increased their activity in Fe-deficient *Beta vulgaris* (López-Millán et al., 2001) and the enzyme activities returned to control levels 24 hours after the resupply with Fe. In *B. rapa*

plants grown under Zn deficiency it was noted that FUM activity dropped while MDH activity rose in comparison to controls (*Blasco et al.*, 2015).

In our experiment we found a decline in the portion of the TCA cycle carried out by FUM; and an increase in the part of the TCA cycle carried out by MDH under Zn shortage. The decrease in FUM activity may be caused by a reduction in previous activities within the TCA cycle, or by an enhancement of divergent pathways leading to an exit of fumarate from the cycle, and thus reducing FUM activity. One of these divergent pathways leads to amino acid biosynthesis from 2-oxoglutarate. This pathway could be especially enhanced: as confirm, an increase in aspartic acid was measured under Zn-deficiency. Different was the response in Zn toxic plants where supra-optimal concentrations of Zn did not seem to affect FUM activity respect to the control.

MDH gene expression was higher in leaves of Zn-deficient rice plants but it was lower in the tolerant genotype, suggesting that this activity is not decisive in Zn-deficit tolerance (*Widodo et al.*, 2010). Barley plants exposed to limited and toxic concentrations of Zn showed a great increment of MDH activity compared with control plants. These results are in contrast with the decline of MDH activity observed in *L.sativa* grown under Zn deficiency (Navarro-Leon et al., 2016); but they agree with those described in *L.sativa* exposed to Zn toxic levels, where MDH activity increased (Barrameda-Medina et al., 2014). Thus, MDH shows a prompt reversible activity and can generate oxalate or malate, depending on reductants availability and substrate levels. The malate produced possibly does not enter TCA, thus also explaining the lower CS activity in these plants.

Apparently, the condition in which Si mostly affected TCA cycle was Zn deficiency. Under Zn limitation, plants showed an increase of CS and FUM activity, inducing an accumulation of malate that was favoured by a decrease of MDH activity. Under Zn control and Zn toxicity, the Si effects were not very clear, suggesting a not decisive role of Si in the regulation of TCA Cycle under Zn stress.

5.9 Organic acid

Organic acids are compounds that have a special structure which proves necessary to tolerate stress, as these contain predominantly carbon, hydrogen, and oxygen, and have one or more carboxyl groups (Jones, 1998). Therefore, organic anions may function as chelants that sequester Zn ions in the cytosol and subcellular compartments (Haydon and Cobbett, 2007). In this sense, Sun et al., (2006) have indicated that the accumulation of organic anions in the vacuole against an excess of metals could explain the role of these compounds in the subcellular compartmentalization of these elements under toxicity conditions. Recently, Liu et al., (2012) defined organic anions as chelants and transporters of Zn and therefore necessary for the uptake, translocation, accumulation, and storage within the plant, thereby guaranteeing efficient Zn distribution within the plant and thus detoxification under toxic conditions. Among the organic anions found in plants, coming from the tricarboxylic acid cycle (TCA), the following acids can be distinguished: citrate, aconitate, isocitrate, α -ketoglutarate, succinate, fumarate, malate, and oxalate. All these anions are produced in catalytic amounts in plant tissues, although only citrate, malate, and oxalate are accumulated (Sousa et al., 2009). Different studies indicate that detoxification by organic anions in leaf did not correlate with the different Zn treatments applied, while in root, a positive relation was found in citrate and malate (Zhao et al., 2000) as well as with oxalate (Liu et al., 2012). Confirming this hypothesis, it has recently been confirmed in *Erica andevalensis* plants that tolerance against high Zn appears to be promoted by the immobilization of the metal in the root system together with a low interference with nutrient uptake and increased production of organic ligands such as citrate and aminoacids (Rossini-Oliva et al., 2012). On the contrary, other authors indicate a positive relation of citrate (Salt et al., 1999) or malate (Mathys, 1977; Godbold et al., 1984) in leaves and the resistance to Zn toxicity, although this correlation depends on the plant species and the Zn concentration available in the culture medium. In this sense, by a comparative analysis between *Thlaspi caerulescens* and *Thaspi ochroleucum*, Shen et al. (1997) demonstrated that in the former species (a Zn-accumulating plant and therefore resistant to this trace element) there was a greater Zn concentration in leaf after the application of 500 μ M Zn in comparison to control (10 μ M Zn),

which was associated with a rise in the concentration of malate in leaf, whereas in *T. ochroleucum* (a Zn-sensitive plant) the Zn concentration increased more in root with respect to control in comparison with *T. caerulescens*, with higher citrate levels in leaf in *T. ochroleucum* than in *T. caerulescens*. However, Wójcik et al. (2006), working with *T. caerulescens* detected an increase both in malate as well as in citrate in leaf, the accumulation level depending on the metal concentration. On the other hand, in leaves of beetroot (*Beta vulgaris*), showing a great capability to accumulate heavy metals, Sagardoy et al. (2011) demonstrated that the citrate and malate concentrations changed at the level of the leaf according to the Zn concentration used in the experiment. Finally, more recent work (Schneider et al., 2013) with *T. caerulescens* confirms the results of Shen et al. (1997), noting that the application of 500 μM of Zn increased the malate concentration in leaf, permitting the sequestering of Zn in the vacuole and conferring avoidance to the plant without toxicity symptoms in this organ.

Different results have been obtained in studies relating OA concentration in leaves with micronutrient disorders but in most of them there was an increase in citrate concentration in plants grown under different mineral stress conditions: Fe deficiency and Zn toxicity in *Beta vulgaris* (López-Millán et al., 2001; Sagardoy et al., 2011), and Zn deficiency and toxicity in *B. rapa* (Blasco et al., 2015). This is consistent with our results in *H. vulgare* where citrate concentration rose by 15-25% both under Zn deficiency and toxicity, although this increase is not due to greater CS activity in the leaves of these plants, which diminishes under Zn stress conditions: thus, it appears that citrate concentration is affected in the same way by both Zn toxicity and Zn deficiency. Therefore, citrate could help barley plants to accumulate more Zn under Zn shortage, or to store it under Zn toxicity conditions. This is supported by the fact that citrate is a strong chelator with high mineral-binding capacity and it could contribute to homeostasis in plants under mineral stress (Sagardoy et al., 2011).

Malate is another OA that can be affected by Zn concentration, as reported in tolerant genotypes of rice to Zn deficiency in which this OA rose both their concentration and their efflux from roots

(Rose et al., 2011). Nevertheless, in other studies malate was not decisive for better tolerance to Zn stresses as observed in *B. rapa* plants where it declined (*Blasco et al.*, 2015). On the other hand, in leaves of *B. oleracea* plants submitted to Zn toxicity it has been reported an increase in malate concentration and it has been suggested that it could help to transport Zn to aerial tissues under Zn-toxicity conditions (*Barrameda-Medina et al.*, 2014). According the results of the present study malate did not play a significant role under Zn deficiency in barley plants; on the other hand, Zn toxicity increased the concentration of malate in leaves. This result can be explained by a higher MDH activity in these plants, not necessarily participating to TCA, but increasing MA to OAA interconversion to satisfy the increased reductant request under Zn stress conditions. Under this view, the changes observed under Si supply suggest that Silicon is able to promote a OAA/MA balance more favorable to OAA, inducing an increased production of reductants able to reduce stress effects and ameliorating the response to Zn limitation or excess. This would also explain the low CS activities in these plants.

There are no studies showing a relationship between oxalate and Zn-deficiency tolerance, but in a study by *Mathys* (2006) it was detected that applying Zn to the nutrient solution, the synthesis of oxalate was enhanced in Zn-resistant, but inhibited in Zn-sensitive ecotypes of *Silene cucubalus* and *Rumex acetosa*. By contrast, it has been observed in both *L. sativa* and *B. oleracea* that oxalate was not decisive for a greater tolerance under Zn toxicity conditions (*Barrameda-Medina et al.*, 2014).

However, malate/oxalate ratio could be very important to face Zn stress in barley plants. Particularly, in Zn deficient plants, the great increase in oxalate levels could be due to the huge increase in MDH activity which mainly reacts to oxidise malate to oxalate, thus increasing the production of NAD(P)H to contrast stress effects. Furthermore, the oxalate increase is supported by a study that demonstrated that the reaction of Zn with oxalate forms a stable Zn-oxalate complex (*Sillen*, 1964), thus oxalate might join Zn in the soil forming Zn-oxalate complexes that would be absorbed by plants in soils with low Zn bioavailability.

Si effects on organic acid seemed to confirm the beneficial effect of the silicate in Zn stress tolerance: under Zn deficiency, Si application increase malate and oxalate probably helping the Zn uptake and transport to the shoots; under Zn toxicity, Si induced a decrease of citrate concentration while unaffected malate levels possibly reducing Zn uptake.

Silicon supply induced a strong increase of oxalate levels thus strongly decreasing the malate/oxalate ratio, and therefore causing a possible strong increase of reductants able to counteract the oxidative stress provoked by both Zn deficiency and excess. It should be underlined that under Zn deficiency the malate/oxalate ratio was 85, and Si supply reduced this ratio to 62; under Zn excess the same value decreased from 108 to 80.

6 CONCLUSIONS

Plants exposed to stress conditions reduce their growth since they invest their energy in counteracting the stress. Barley plants exposed to Zn deficiency and toxicity showed the classic symptoms of stress: an inhibition of growth representing by a decrease of relative growth rate and biomass.

Zn is required as structural and catalytic components in many proteins and enzymes for normal growth and development. Furthermore, Zn is very closely involved in the N metabolism of plants: our results clearly indicated that Zn stress severely affected N metabolism. Low and high Zn doses reduced plant capability to uptake nitrogen but enhanced the efficiency in N utilization. Zn did not seem to affect NR activity that stayed stable to control levels but increased NH_4^+ concentration as a consequence of an enhancement of photorespiration. In contrast with the stability of NR activity and the inhibition of N uptake, NO_3^- content increased under Zn stress, probably because reductants in leaves diverted from basal metabolism to enhance stress response. NH_4^+ is highly toxic in plants, so the high NH_4^+ levels were quickly absorbed by an enhanced GS/GOGAT cycle. The concentration of NH_4^+ was increased, so that GDH also participated in the assimilation of N despite the presence of GS activity. AAT activity showed a similar pattern respect to GS activity and this could suggest that the Zn stress negative effects on biomass of the aerial part could be due to a greater amino acids synthesis by AAT activity but not so efficient to contrast the stress induced by the micronutrient. Photorespiratory nitrogen cycle contributes to the metabolism of certain amino acids so we supposed that enhanced GGAT activity cooperatively with other photorespiration enzymes induced an increase in the amino acids production. In fact, Zn deficiency or toxicity condition promoted an increased in the total amino acid concentration.

Under Zn stress condition, plants showed Pro and GB increment that not represented a signal of resistance to toxicity, but probably a symptom of Zn stress.

SPAD values correlated well with plant phenotypes since plants submitted to low Zn and high Zn doses presented more chlorotic aspect and lower SPAD values, and the opposite for plants submitted to control Zn dose with higher SPAD values and a greener phenotype.

Zn deficiency and toxicity clearly affected carboxylate metabolism. Firstly, PEPC activity was enhanced, suggesting a possible strategy to synthesize more citrate and oxalate. Furthermore it was observed an increase in the part of the TCA cycle carried out by MDH, and the malate produced possibly did not enter in TCA, explaining the lower CS activity in these plants; and a decline in the portion of the TCA cycle carried out by FUM under Zn shortage, possibly because of an exit of fumarate from the cycle for divergent pathways as amino acid biosynthesis from 2-oxoglutarate. Citrate concentration rose probably to help plants to accumulate more Zn under Zn shortage, or to store it under Zn toxicity conditions. Zn toxicity increased malate, as suggested by a higher MDH activity. The increased oxalate levels could be due to the enhanced MDH activity which mainly reacts to oxidise malate to oxalate. Not-optimal Zn nutrition negatively affected barley plants also increasing the oxidative stress as shown by an accumulation of O_2^- and H_2O_2 and a diminution in total Chls and carotenoids concentrations. The high H_2O_2 accumulation could be explain by the unincreased activity of APX. Zn stress increased levels of CAT activity and intensified GR as a possibly mechanism to ameliorate stressful conditions by sustaining the pool of reduced glutathione. The decrease in the ratio reduced/oxidized forms of the two antioxidants, AsA and GSH, and in their redox status confirmed the intensification of the ascorbate-glutathione cycle.

The negative Zn stress effects appeared to be mitigated by Si in our experiment. The addition of Si to plant nutrition, levelled leaf biomass and RGR to values similar to the levels observed under optimal conditions without Si. These plants responses could be interpreted as a positive effect of Si, in reducing the growth limitation induced by Zn shortage or excess. Si acted ameliorating stressed plants also affecting N metabolism. Under different Zn nutrition, Si potentiated NU_pE and did not affect NU_tE, inducing an increment of nitrogen forms (NO₃⁻, N organic and TNC). Zn suboptimal concentration combined with Si enhanced NR and kept stable NH₄⁺ levels since it was consumed by potentiated GDH activity. Differently, supra-optimal Zn concentration combined with Si, inhibited NR activity, incrementing NO₃⁻ and accumulated NH₄⁺ probably for an increment of GDH deamination activity. Furthermore, in Zn stress conditions, Si seemed to inhibit photorespiration and increased proline accumulation that acted as a stress sensor more than a stress effect. Si affected TCA cycle particularly under Zn deficiency, increasing CS and FUM activity and inducing an accumulation of malate that was favoured by a decrease of MDH activity.

The beneficial effects of Si on stress tolerance were confirmed by a diminution of ROS levels possibly caused by a better photosynthesis performance and an inhibited APX activity. Si induced in Zn stressed plants a decrease in oxidized form and an increase in reduced form of the antioxidant compounds (AsA and GSH).

Under Zn control condition, Si decreased biomass and RGR showing that Si negatively affected these plants. Furthermore, inhibited nitrogen uptake efficiency but improved the nitrogen utilization. In these plants, Si application increased photorespiration rate and proline content.

The not beneficial Si effects were again confirmed by ROS accumulation and a decrease of the reduced ascorbate and glutathione.

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Chapter 5

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Early responses to Cadmium exposure in barley plants: effects on biometric and physiological parameters

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Key Words

Nitrate reductase, G6PDH, Amino acid levels, Heat shock proteins, APX, RWC.

Chapter 5

Early responses to Cadmium exposure in barley plants: effects on biometric and physiological parameters

Abstract

BACKGROUND: Cadmium represents one of the most toxic pollutants in plant ecosystems: at high concentrations it can cause severe effects, such as plant growth inhibition, decrease in photosynthesis and changes in plant basal metabolism.

RESULTS: Changes in pigments content, RubisCO large subunit (LS), and D1 protein indicated a severe reduction in photosynthetic efficiency. Furthermore, the decrease of nitrate reductase activity and changes in free amino acids levels show a general stress condition of nitrogen assimilation.

Cadmium increased the activities of ROS-scavenging enzymes; among these, ascorbate peroxidase rate was the most noticeably increased. It is worth noting that glucose-6-phosphate dehydrogenase (G6PDH; EC 1.1.1.64), showed changes in both activities and occurrence during cadmium stress.

Interestingly, our data suggest that G6PDH would modulate redox homeostasis under metal exposure, and possibly satisfy the increased request of reductants to counteract the oxidative burst induced by cadmium.

CONCLUSION: Therefore, the results suggest that APX and G6PDH may play a pivotal role to counteract the oxidative stress induced by cadmium in young barley plants.

Introduction

In recent years, heavy metals accumulation in soils has become a critical constraint for crop cultivation. This pollution is caused by industrial activities, irrigation using wastewaters, excess of pesticides, herbicides, phosphate fertilizers, and other chemicals (Yousaf et al. 2016; Akhtar et al. 2017; Kintlova et al. 2017).

Among heavy metals, cadmium (Cd) is present worldwide in soils both from natural sources and anthropic pollution (Choppala et al. 2014; Akhtar et al. 2017). Cd does not play any biological function and exhibits high toxicity even at low concentrations, disturbing many aspects of plant physiology, by reducing total biomass, fresh and dry weight, root length and leaf size, in both hydroponics and soil cultivation (Yousaf et al. 2016; Akhtar et al. 2017; Rizwan et al. 2017).

It has been widely shown that Cd stress affects vegetables quality and production: potato (*Solanum tuberosum*) exposed to Cd exhibited smaller tubers; tomato (*Solanum lycopersicum*) showed a significant reduction in chlorophylls and carotenoids, affecting the plants photosynthetic capability and resulting in no fruit production in the presence of 100 μM Cd (Rizwan et al. 2017).

Cd uptake decreases photosynthesis, conductance and water use efficiency (Rizwan et al. 2017; Zemanova et al. 2017). The reduction in chlorophylls and carotenoids contents was reported in various crops, as well as different changes in metabolite concentrations, including significant modification in amino acids and organic acids (Rizwan et al. 2017; Zemanova et al. 2017).

Cd induces severe oxidative stress by increasing levels of reactive oxygen species (ROS), such as superoxide anion radical ($\text{O}_2^{\circ-}$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($^{\circ}\text{OH}$) (Sandalio et al. 2001). Therefore, the detoxification of ROS by scavenging enzymes plays a critical role (Gill and Tuteja 2010; Landi et al. 2017).

Cadmium exposure in plants enhanced the activities of enzymes such as catalase (CAT), superoxide dismutase (SOD), glutathione reductase and guaiacol peroxidase (GPX) (Leon et al. 2002). On the other hand, enzymes such peroxidase (POD) and ascorbate peroxidase (APX) play diversified roles depending on species or even varieties (Gill and Tuteja 2010; Rizwan et al. 2017). Particularly, the ascorbate-glutathione cycle plays a pivotal role, maintaining an adequate photosynthetic rate even under metal stress (Gill and Tuteja 2010). Particularly, APX occurrence, activities and expression were stimulated in crops upon heavy metal stress, drought, flooding and salinity, highlighting the crucial role of APX as ROS scavenger (Gill and Tuteja 2010; Iovieno et al. 2016; Landi et al. 2017).

More specifically, a critical role in those response mechanisms triggered during oxidative burst has been defined in different crops for glucose-6-phosphate dehydrogenase (G6PDH – EC 1.1.1.49), (Yang et al. 2014; Landi et al. 2016). The reducing power produced by G6PDH activity as NADPH plays a critical role in plant physiology participating in biosynthetic pathways (e.g. ribose for nucleotides biosynthesis) (Castiglia et al. 2015; Esposito 2016); nitrogen assimilation (Esposito et al. 2005); and required by the ROS scavenging systems under abiotic stress (Cardi et al. 2011; Dal Santo et al. 2012; Cardi et al. 2015; Landi et al. 2016).

Barley (*Hordeum vulgare*) represents a primary resource for food and forage demand (Landi et al. 2017) and is the fourth most important cereal crop worldwide (Shen et al. 2016). Furthermore, barley is an important cereal model, presenting genomics and bioinformatics resources including complete genome sequence, transcriptome under various environmental stresses, and several on-line databases (The international barley genome sequencing consortium 2011; Kintlova et al. 2017). Among cereals, barley shows a natural abiotic stress tolerance: namely, cultivated barley (*Hordeum vulgare*)

retains a 40% alleles compared with the original barley progenitor (*Hordeum spontaneum* – Ellis et al. 2000). This wild ancestor showed remarkable tolerance to salt, drought and heavy metals stress, but breeding programs have produced high-yielding barley cultivars more sensitive to abiotic stress, making this aspect a critical issue in barley as well (Shen et al. 2016; Landi et al. 2017).

The aim of this work is explore the common responses of barley plants exposed to cadmium and elucidate the possible role(s) of G6PDH and other enzymes (ascorbate peroxidase – APX and nitrate reductase - NR) under heavy metal stress.

To this purpose, barley plants were exposed to the presence of toxic levels of Cd, and enzymatic activities, HSP70s, levels of pigments, photosynthetic efficiency, photosynthesis- related proteins, and free amino acids were determined. The possible mechanisms of Cd response in barley will be finally discussed.

Material and methods

Plant material and stress treatments

Seeds of barley (*Hordeum vulgare*, var. Nure), were supplied by “Centro di ricerca per la genomica e la postgenomica animale e vegetale” (CRA-GPG – Fiorenzuola D’Arda – PC, Italy). Seeds were germinated for 5 days in the dark on moistened paper, then seedlings were grown in hydroponic solution in darkened plastic bottles at 20°C, at 60-80% relative humidity, under 16h-light/8h-dark regime, with approximately 180 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$. The composition of the medium (modified Hoagland solution), continuously bubbled with air, has been previously described (Cardi et al. 2015).

After 10 days of hydroponic culture, plants were divided in two groups: control plants were maintained in the standard growth medium; Cd stressed plants were grown in the presence of 10^{-5} M of CdCl_2 added to the standard medium. Nutrient solutions were

controlled for pH and daily adjusted. Leaves and roots from each group of barley plants were collected at 0h, 6h, 24h, 48h and 7 days after the stress induction.

Growth variation and water content determination

Changes in length of barley leaves and roots exposed to Cd were measured at the end of the experimental design on five randomly chosen plants. To measure the Relative Water Content (RWC), 15-20 plants were weighted from Cd exposed and control groups soon after hydroponic growth to determine FW. The plants were hydrated for 3-4 hours by either floating in a Petri dish in distilled water and weighed to determine the turgid weight (TW); then samples were dried overnight at 70°C for the calculation of the dried weight (DW). The plant RWC was derived from the formula:

$$\text{RWC \%} = (\text{FW}-\text{DW})/(\text{TW}-\text{DW}) * 100 \text{ (Matin et al. 1989).}$$

Photosynthetic parameters measurements

Photosynthetic parameters: Maximal photochemical efficiency (Fv/Fm); Photosystem II quantum yield (QY); electron transport rate (ETR) and non-photochemical quenching (NPQ) were determined using a portable PAR-FluorPen FP 100-MAX-LM fluorimeter equipped with a light sensor (Photon System Instruments, Czech Republic) on young barley plants exposed to 7d to 10⁻⁵M Cd using the default settings, as previously described (Arena et al. 2017).

Chlorophyll and carotenoids content

Chlorophyll and carotenoids were assayed using the method reported by Hu et al. (2013). Samples of barley leaves (500 mg) were suspended in 1.5 ml of N-N'-dimethyl-formamide (Sigma-Aldrich) and incubated 4h at 4°C in the dark. Pigment levels were

calculated by measuring absorbance at 664, 647 and 480 nm, using optical glass cuvettes (OG-6040 Hellma) in a Cary 60 spectrophotometer (Agilent Technologies, USA) according to Moran (1982).

Proline content

Proline content was determined using the method of Claussen (2005). Finely ground leaf and root tissue (250 mg) was suspended in 1.5 ml of 3% sulphosalicylic acid, and filtered through a layer of glass-fiber filter (Macherey-Nagel, Ø 55mm, Germany). 1ml of glacial acetic acid and 1mL ninhydrin reagent (2,5 g ninhydrin/100 mL of a 6:3:1 solution of glacial acetic acid, distilled water and 85% ortho-phosphoric acid, respectively) were added to 1 mL of the clear filtrate. After incubation at 100°C for 1 h, optical density of samples was read at 546 nm in a Cary 60 spectrophotometer (Agilent Technologies, USA).

Glucose-6-phosphate dehydrogenase assay

G6PDH was extracted by grinding 300 mg of barley leaves and roots, suspended in 600 µl of solution containing 50 mM Tris-HCl at pH 8.0, 5 mM MgCl₂, 4 mM EDTA, 10% glycerol, 15 µM NADP⁺, and 10 µl of Protease Inhibitor Cocktail (Sigma P9599) for ml of extraction solution.

G6PDH activity was assayed as described previously (Castiglia et al. 2015), by monitoring NADP⁺ reduction at 340nm using a Cary 60 spectrophotometer (Agilent Technologies, USA). The assay mixture contained: 50 mM Tris-HCl pH 8.0, 50 mM MgCl₂, 1.5 mM NADP⁺, 30 mM glucose-6P, and extract (10-100 µl). Activity was expressed as nmol reduced NADP⁺ min⁻¹ mg⁻¹ protein.

Western blotting

For Western blotting analysis, proteins were extracted as described previously and separated by SDS-PAGE (Cardi et al. 2011). Then polypeptides were transferred onto a Hybond nitrocellulose membrane (GE Healthcare). The filter was incubated with primary antibodies vs G6PDH (Cyt, P1 and P2) (Wendt et al. 2000;, 2015; Castiglia et al. 2015), HSP70 (Cyt, Chloroplastic and mitochondrial - Agrisera), D1 and RuBisCO large subunit (Agrisera). After incubation of the membrane with secondary antibodies, cross-reacting polypeptides were identified by enhanced chemiluminescence using the ECL Prime kit (GE Healthcare). Images were acquired by BioRad Chemidoc system (Bio-Rad, USA).

Ascorbate peroxidase assay

APX activity was assayed by monitoring the ascorbate oxidation at 290 nm, using a Cary 60 spectrophotometer (Agilent Technologies, USA). APX was extracted from 300 mg of leaf and root tissue suspended in 600 μ l of solution containing 50 mM NaH_2PO_4 buffer (pH 7.0), 2 % PVPP, 0.1 mM EDTA, 2 mM. The reaction mixture contained 30 mM KH_2PO_4 buffer (pH 7.0), 0,1 mM EDTA, 0,5 mM Na-ascorbate and 0,06% H_2O_2 (Nakano and Asada 1981).

Nitrate reductase assay

Nitrate reductase (NR) was extracted from leaf and root tissue (1 gr.) suspended in 2 ml of solution containing 25 mM NaH_2PO_4 buffer (pH 7.2); 1 mM cystein; 25 μ M FAD.

The assay mixture contained 25 mM NaH_2PO_4 buffer (pH 7.2); 1 mM benzyl-viologen; 0.2 M KNO_3 ; sodium dithionite (8mg/ml in 25 mM NaH_2PO_4); and 200 μ l of extract.

The reaction mixture was incubated for 20 minutes at 30°C; then a solution containing

barium acetate 0.15 M, ethanol 95% was added to stop enzymatic reaction. The tubes were then vigorously shaken, centrifuged; nitrite formed by NR was estimated colorimetrically at 540 nm in a Cary 60 spectrophotometer (Agilent Technologies, USA) with the addition of a mixture containing 0.67 ml sulphanilamide (4% in 3 N HCl) and 0.33 ml N (1 – naphthyl) ethylenediamine dihydrochloride (0.08% in water) (Vona et al. 2004). Nitrate reductase activity was expressed as nmol nitrite produced $\text{min}^{-1} \text{mg}^{-1}$ protein.

Amino acids determination by high performance liquid chromatography (HPLC)

Soluble amino acids were extracted in 0.85 ml of 80% ethanol for 15 min at 4°C, and then centrifuged (Eppendorf 5415D) . The supernatant was filtered using Waters Sep-Pak C18 Light Cartridges. An aliquot (50 μl) of the extract was derivatized for 1 min with *orto-phthaldialdehyde* (oPA) and separated by HPLC for amino acid analysis. Chromatographic equipment was Agilent 110 HPLC series. The oPA derivatives were separated on a reverse-phase C18 ultrasphere column (250 mm \times 4.6 mm). Solvent A consisted of 50 mM Na-Acetate (pH7), 1% tetrahydrofuran, and solvent B was absolute methanol (Carlo Erba). A sample (20 μl) of the mixture was injected and eluted at a flow rate of 1 ml min^{-1} . The eluted oPA derivatives were detected by a variable wavelength detector (Agilent G1314A). Quantification of single amino acids was made against a relative calibration curve and expressed as $\mu\text{mol g}^{-1}$ FW (Barrameda-Medina et al. 2017).

Statistics

Values were expressed as mean \pm standard error (SE). Each experiment was made in at least three replicates. Statistical significance of biometric parameters, water content,

HPLC experiments and physiological analysis between control and Cd exposed plants was evaluated through Student's t-test ($p \leq 0.05$). In addition, a one-way analysis of variance (ANOVA) was used to calculate the statistical significance of the differences occurred in enzymatic activities (G6PDH, APX and NR) and proline content between controls and Cd exposed groups in different collection points (ANOVA calculations correspond to $\alpha = 0.05$). Differences between means were evaluated for significance using the Tukey–Kramer post-hoc test.

RESULTS

Cd exposure induces a severe stress in barley plants

The effects of Cd stress were observed by exposing barley plants to 10^{-5} M of CdCl_2 for 7 days: metal-exposed plants exhibited wilting and leaf curling (Figure 1A); Cd stress induced a severe loss in plant biomass and growth (Supplemental Figure 1), resulting in a 42% and 47.5% decrease in leaves and roots length, respectively (Figure 1B). Furthermore, Cd stressed plants showed a significant reduction in relative water content (%RWC), from 6h (-12.2%) to 1d (-19.1%), 2d (-16.3%) and 7 d (-17% - Figure 1C).

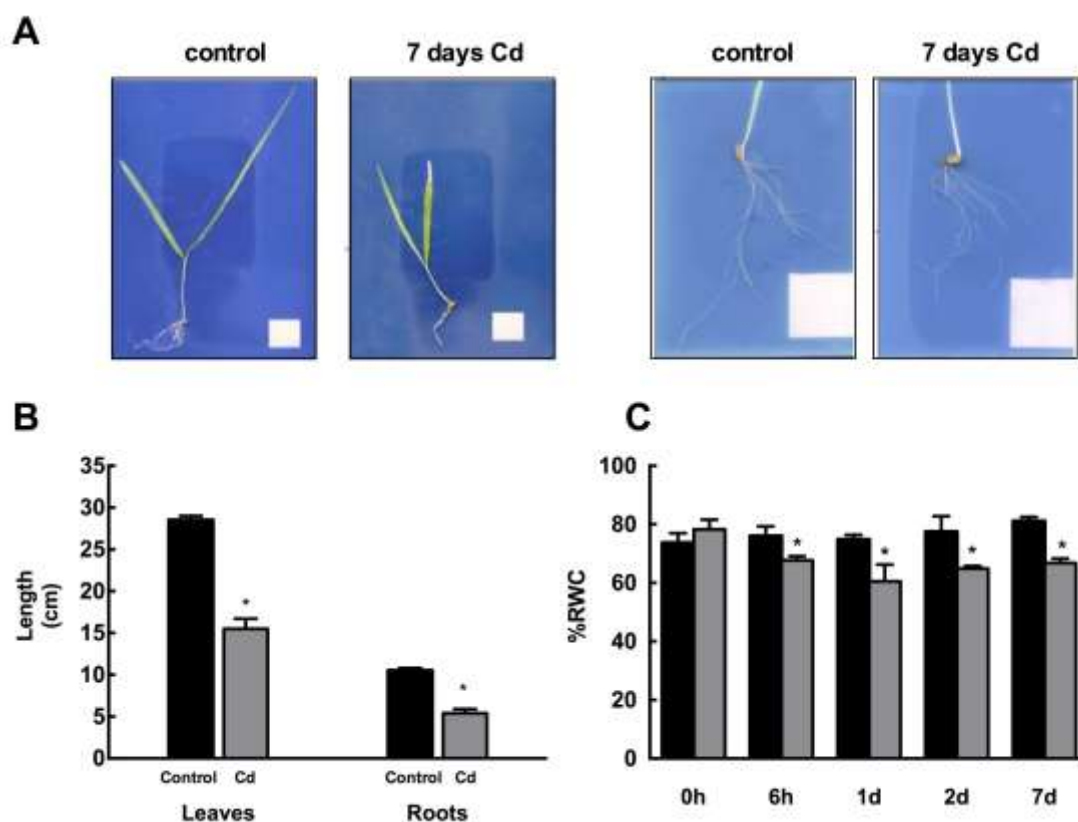


Figure 1 – Biometric effects of Cd on barley plants. Effects of 7 days Cd exposition on barley leaves and roots (A). Changes in leave and root length (B) in control (no Cd - black bars) and stressed (grey bars) barley plants after 7 days of cadmium exposition. Changes in relative water content (%RWC) in controls (no Cd - black bars) and stressed (grey bars) barley plants (C). Asterisks indicate significantly different values in Cd stress vs control plants at $p \leq 0.05$ (*).

Cd exposure induced the accumulation of stress related proteins such as HSP70s. As shown in figure 2, a prompt increase in cytosolic, chloroplastic and mitochondrial HSP70s levels was reported in leaves after 6h, remaining higher than control values during the entire experimental period (Figure 2A). On the other hand, a lower but similar increase in cytosolic and mitochondrial HSP70 was detected in barley roots exposed to Cd (Figure 2B).

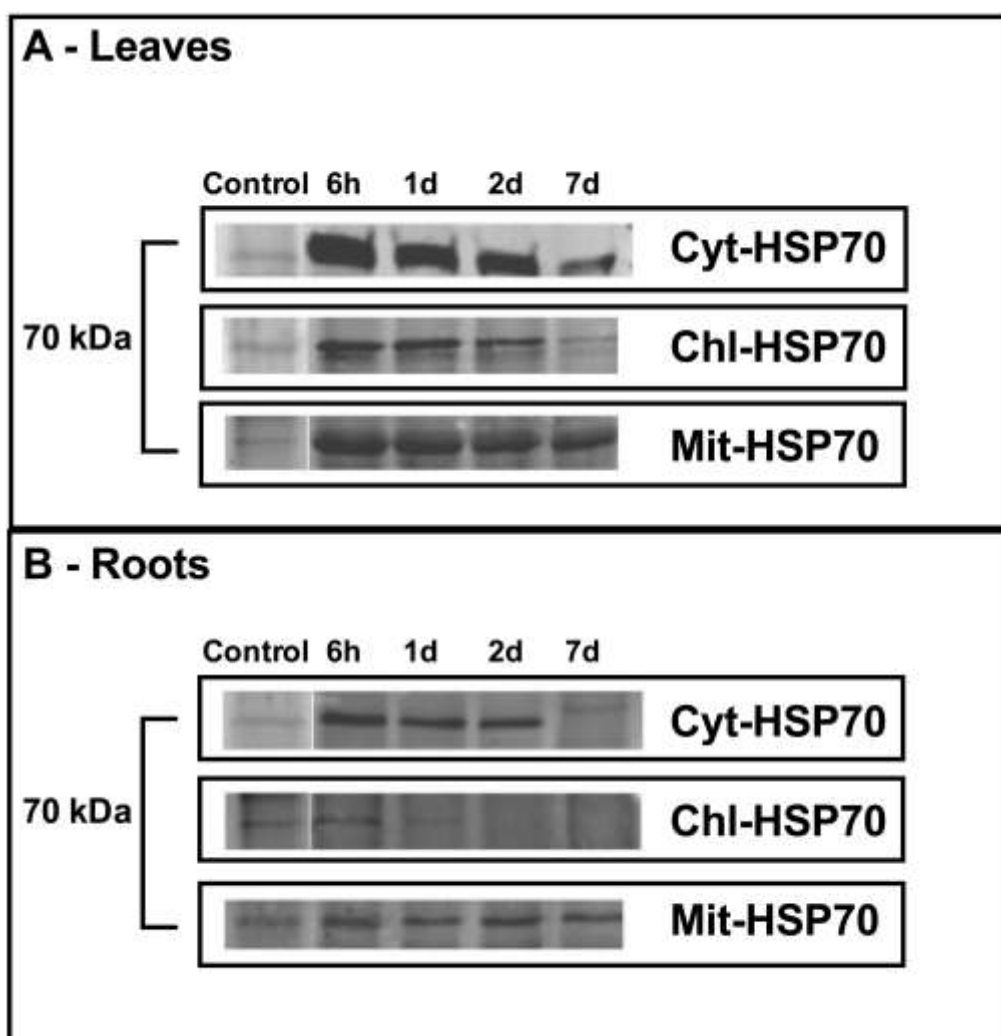


Figure 2 - Occurrence of HSP70 measured by Western blotting - using anti-HSP70 antibodies (Agrisera) - in leaves (A) and roots (B) of barley plants exposed to 10^{-5} M Cd for 6h, 1day, 2 days and 7 days.

In order to verify possible changes in osmoregulation mechanisms, proline content was measured in the leaves. Cd stressed plants showed significant and progressive increases in proline content from 1.8-fold after 6h up to 2.4-fold change after 2d, respectively: barley leaves exposed to Cd for 2 days showed a proline content of 2.64 mg gr⁻¹ FW (Figure 3A). Contrarily, barley roots exposed to Cd showed a significant increase in proline content after 6h (+60%), then proline content decreased to the levels of control plants (Figure 3B).

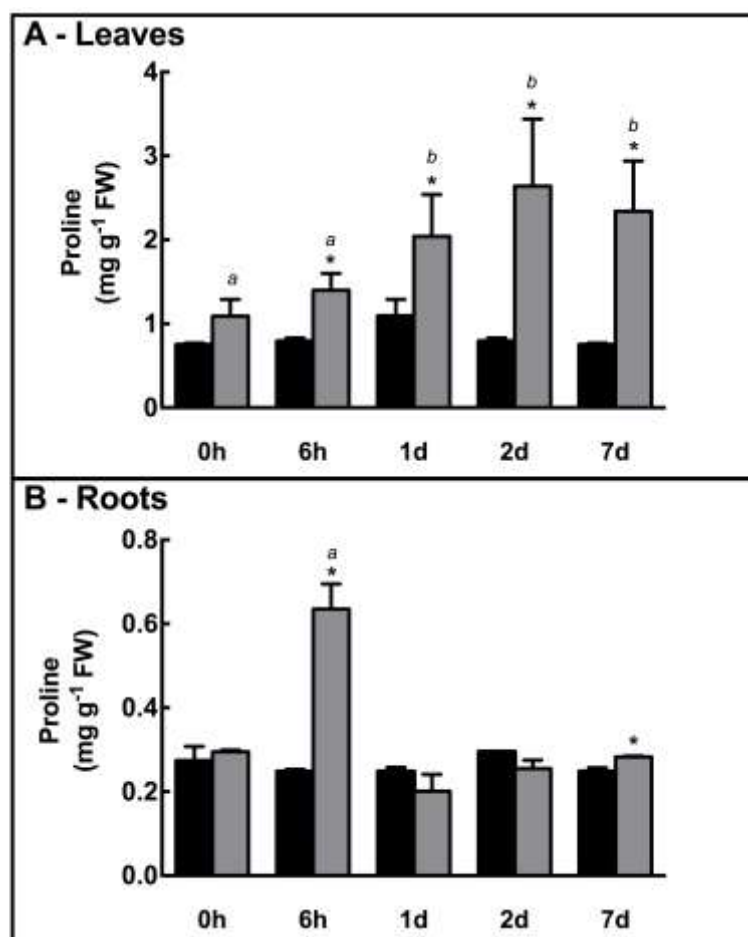


Figure 3 - Changes in the levels of proline in control (no Cd - black bars) and 10⁻⁵M Cd-stressed (grey bars) barley leaves (A) and roots (B). Asterisks indicate significantly different values in Cd stress vs control plants at $p \leq 0.05$ (*). Differences between stressed groups were indicated by letters “a” (6h).

Effects of Cd on photosynthesis

Photosynthetic machinery was monitored by measuring changes in photosynthetic pigments, RuBisCO large subunit (LS), and D1 protein of photosystem II. As shown in figure 4A, critical decreases in chlorophyll A, chlorophyll B and carotenoids were reported in leaves exposed to Cd. In detail, a significant decrease about 35% and 27% was reported after 7d for chlorophyll A and chlorophyll B upon Cd stress, respectively. Furthermore, was reported a not-significant 60% reduction in carotenoids content as well.

Western blotting analysis suggested an increase in occurrence of both RuBisCO and D1 proteins (Figure 4B). These results were confirmed by the data on photosynthetic efficiency parameters: HM exposure resulted in a significant slight decrease in Fv/Fm (-6.3%), QY (-5.4%) and ETR (-8.7%). Contrarily, and a 2.5-fold increase in non-photochemical quenching (NPQ) was reported (Table 1).

Table 1 – Effects of Cd stress on photosynthetic parameters in barley leaves. Maximal photochemical efficiency (Fv/Fm); Photosystem II quantum yield (QY); electron transport rate (ETR) and non-photochemical quenching (NPQ) were calculated as described in Materials and methods in barley plants exposed for 7d to 10⁻⁵M Cd. Each value represents the mean ± standard error; n = 5. Asterisks indicate significantly different values in Cd stress vs control plants at p ≤ 0.05 (*).

	Photosynthetic efficiency			
	Fv/Fm	QY	ETR	NPQ
Control	0.79 ± 0.01	0.75 ± 0.01	36.7 ± 1.45	0.07 ± 0.01
Cd (7d)	0.74 ± 0.02*	0.71 ± 0.01*	32.9 ± 0.86*	0.17 ± 0.02*

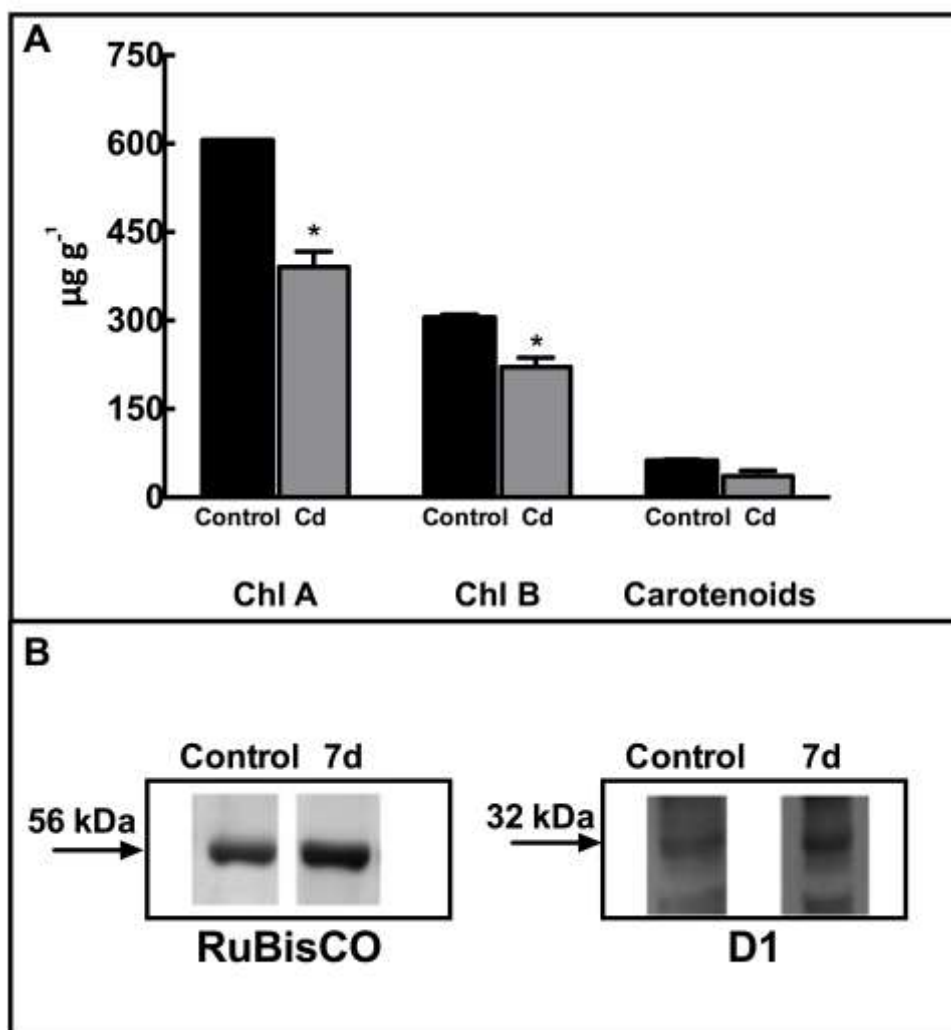


Figure 4 - Changes in the levels of chlorophylls A, chlorophylls B and carotenoids in barley plants exposed to 10^{-5} M Cd for 7 days (A). Levels in Cd-stressed plants are in grey bars; controls (no Cd - black bars). Asterisks indicate significantly different values in Cd stress vs control plants at $p \leq 0.05$ (*) and $p \leq 0.005$ (**), respectively.

(B) Occurrence of D1 and RuBisCO measured by Western blotting using anti-D1 and anti-RuBisCO antibodies (Agriser) in leaves of barley plants exposed to 10^{-5} M Cd for 7 days.

G6PDH and APX exhibit a critical role during Cd stress

The activities and the occurrence of G6PDH isoforms were investigated in barley plants exposed to 10^{-5} M cadmium: this resulted in a prompt 32.5% increase in G6PDH activity with respect to the control plants' after 6 hours in leaves. After 1 and 2 days of cadmium exposure, G6PDH activity remained steadily increased about 36.1% and 27.4% in stressed plants than controls. The peak of G6PDH activity was observed after 7 days of treatment, when the activity increased up to $51 \text{ U mg}^{-1} \text{ prot.}$ (Figure 5A). The ANOVA analysis showed no significant difference between control plants from 0h to 7 days, while statistical changes were reported between the first two days and 7 days Cd-stressed plants.

Furthermore, western blotting analyses revealed slight changes in the occurrence of the different G6PDH isoforms upon Cd stress: a light increase in the occurrence of cytosolic isoform with respect to the plastidial/chloroplastic isoforms was observed (Figure 5B).

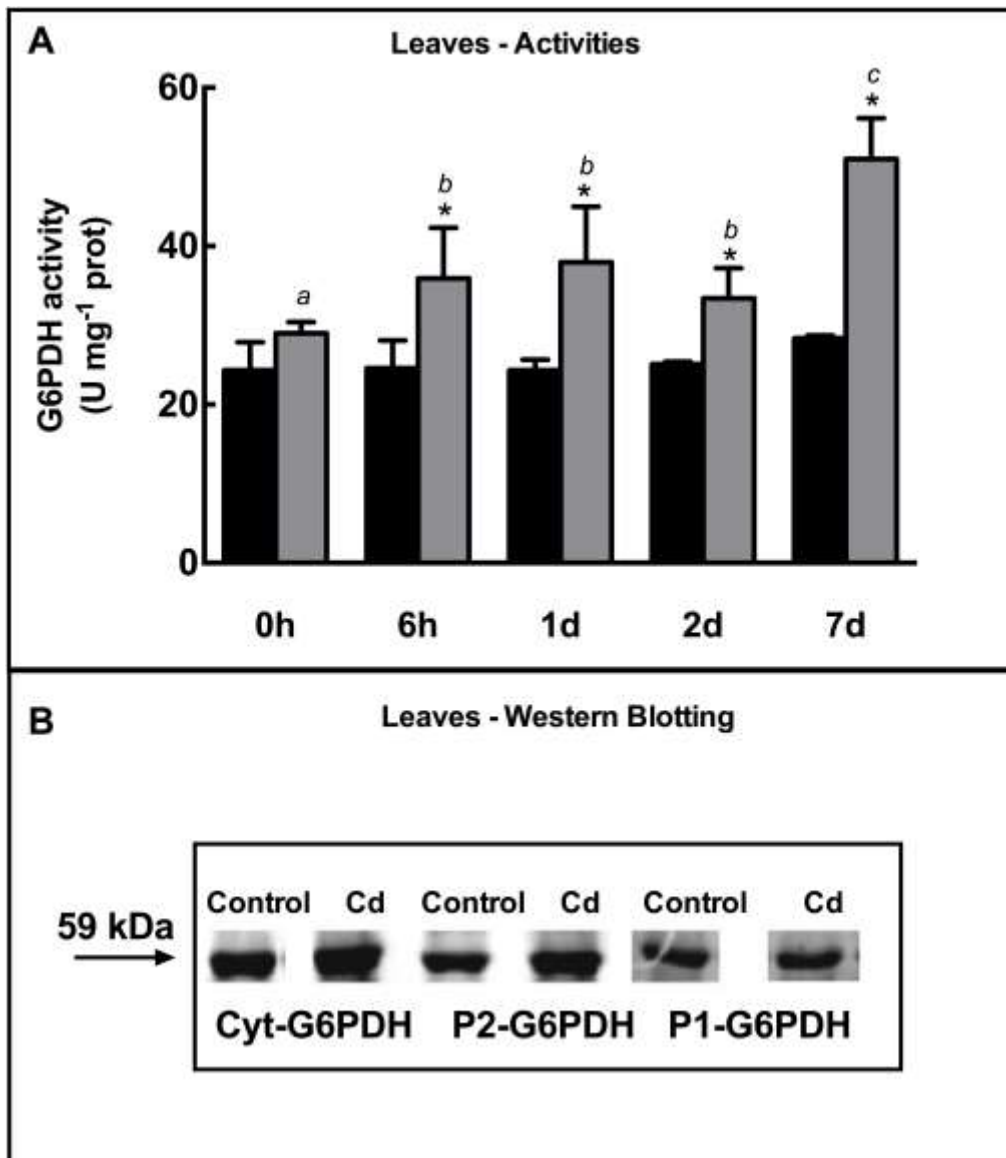


Figure 5 - (A) Effects of Cd stress on G6PDH enzymatic activity in control (no Cd - black bars) and Cd-stressed (grey bars) barley leaves. Asterisks indicate significantly different values in Cd stress vs control samples at $p \leq 0.05$ (*). Differences between stressed groups were indicated by letters “a” (0h), “b” (6h, 1d and 2 d) and “c” (7d). (B) Accumulation of cytosolic, chloroplastic and plastidial G6PDH revealed by Western blotting using anti potato cyt-, P1- and P2-G6PDH antibodies.

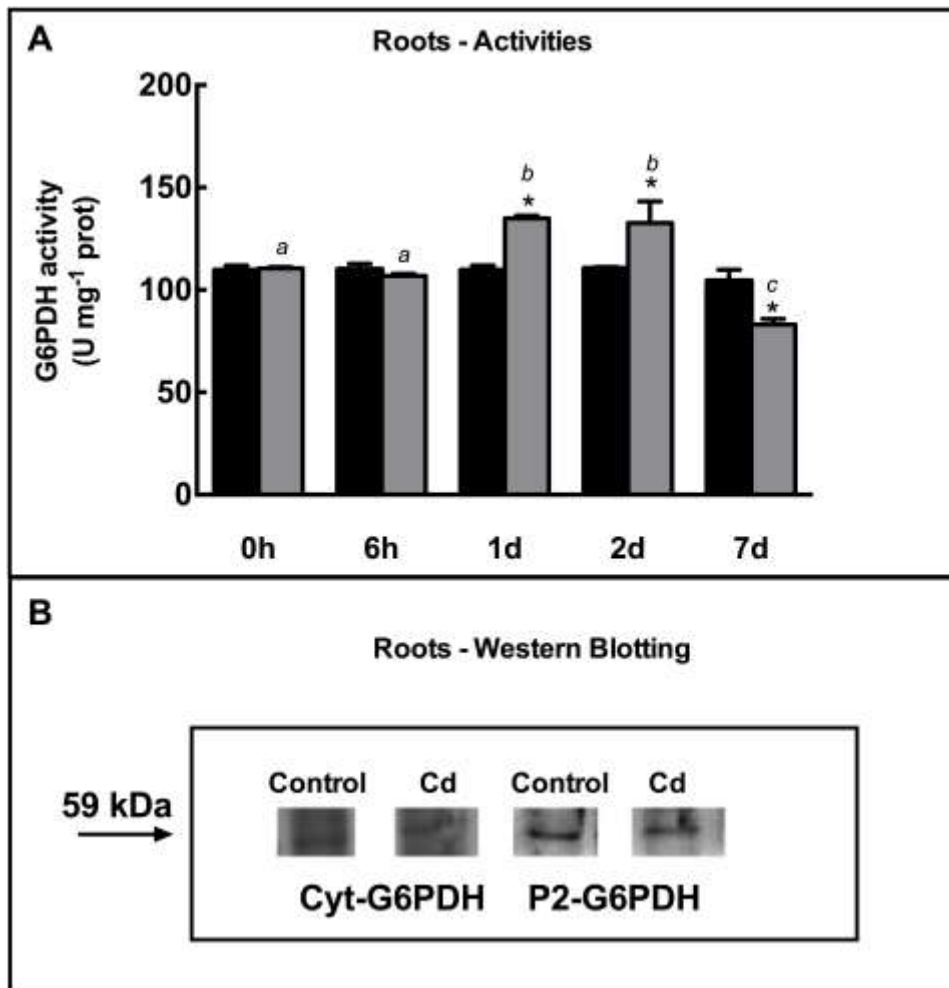


Figure 6 - (A) Effects of Cd stress on G6PDH enzymatic activity in control (no Cd - black bars) and Cd-stressed (grey bars) barley roots. Asterisks indicate significantly different values in Cd stress vs control samples at $p \leq 0.05$ (*). Differences between stressed groups were indicated by letters “a” (0h and 6h), “b” (1d and 2 d) and “c” (7d). (B) Accumulation of cytosolic, chloroplastic and plastidial G6PDH revealed by Western blotting using anti potato cyt-, and P2-G6PDH antibodies.

Interestingly, similar results were observed in roots. A 16-18% increase in G6PDH activity was observed after 1 and 2 days of Cd exposure respectively; then, total G6PDH activity decreased in roots (Figure 6). The ANOVA analysis indicated no significant difference between control plants from 0h to 7 days, while statistical changes were reported between the 1 and 2 days versus 6h and 7 days Cd-stressed plants.

These results were corresponding to those obtained for ascorbate peroxidase activity in the leaves, where the enzyme rate increased about two-fold with respect to the controls within 6 hours of treatment, remaining stable after 1d (+39%) and after 2d (32%), while the APX activity decreased only 7d after stress imposing at control levels (124 ± 2.4 nmol * min⁻¹ * mg⁻¹ prot. - Figure 7A). ANOVA analysis showed no significant differences between control plants from 0h to 7 days. Furthermore, significant differences were reported for 6h and 1 day Cd stressed plants vs 2 days and 7 days Cd stressed plants. Significant differences were also reported in 2 days stressed plants vs 7 days stressed plants. Intriguingly, barley roots exposed to Cd showed significant and rapid increase in APX activities from 6h (+42.6%), to 2 day (+51.6%). After 7 days of Cd exposition APX activity remained higher - up to 121.4 nmol * min⁻¹ * mg⁻¹ prot. - in stressed plants with respect to controls (Figure 7B).

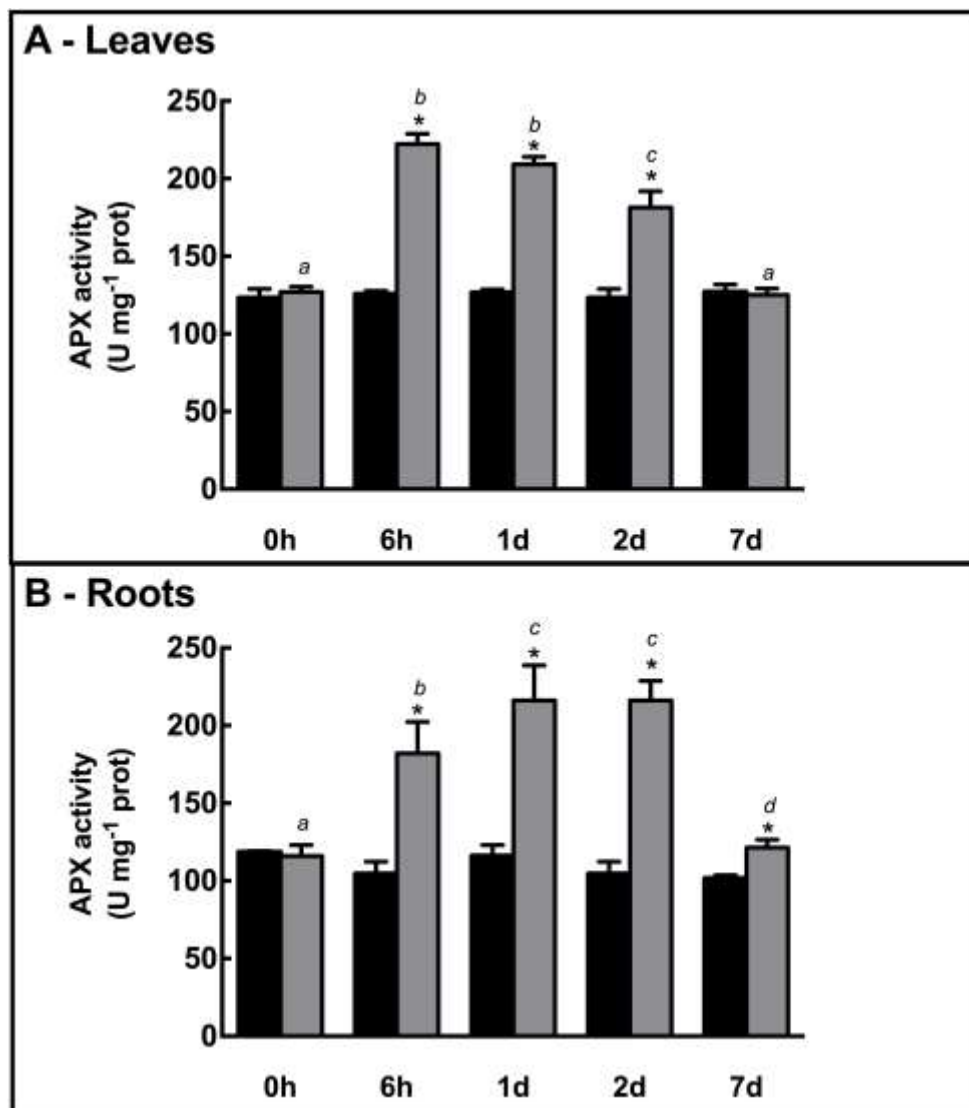


Figure 7 - Effects of Cd stress on APX enzymatic activity in control (no Cd - black bars) and Cd-stressed (grey bars) in barley leaves (A) and roots (B). Asterisks indicate significantly different values in Cd stress vs control plants at $p \leq 0.05$ (*). Differences between stressed groups were indicated by letters “a” (0h and 7 d), “b” (6h and 1d) and “c” (2d) in leaves; Differences between stressed groups were indicated by letters “a” (0h), “b” (6h), “c” (1d and 2d), “d” (7d) in roots.

Effects of Cd on organic nitrogen metabolism

Nitrogen metabolism was monitored by measuring the activity of nitrate reductase. As shown in Table 2, barley plants exposed to Cd exhibited a significant increase in NR activities both in leaves and roots. Barley leaves exposed to Cd showed an activity of $0.05 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ prot.}$ steadily increasing from 1.41 to 2.35-fold change after 1 day and 7 days, respectively; ANOVA analysis showed significant differences between 7 days vs 1 and 2 days leaves from Cd exposed plants.

On the other hand, roots showed an activity of $0.17 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ prot.}$ at 0h; no significant difference was found in control roots during the experimental period.

Cd stress induced a major increase in NR activity of 3-fold change at 2 d of Cd stress. After 7 d the increase remained higher in stressed roots than control ($0.49 \text{ nmol min}^{-1} \text{ mg}^{-1} \text{ prot.}$ – Table 2). ANOVA analysis showed significant differences in 2 days root vs 1 and 7 days roots from Cd exposed plants; further differences were found in 7 days vs 1 day Cd exposed roots.

Cadmium stress induced specific changes in the amino acid levels plants exposed to cadmium (Table 3). Barley roots exposed to Cd showed an increased aspartate content and a correspondent decrease in asparagine and glutamine, almost in a stoichiometric pattern. A not-significant increase in glutamate content was also reported. Severe decreases in serine and valine (-30; -40%) were associated with a increase in alanine levels (+50%). It should be underlined that levels of aromatic amino acids were increased by 20% upon Cd stress, with a particularly marked increase in tryptophan levels (+40%).

In leaves, a significant decrease was observed in aspartate (-20%), and glutamine (-45%), with correspondent increases in asparagine (+7%) and glutamate (+36%). Significant reductions were observed in the levels of arginine (-18%), lysine (-15%) and

valine (-40%). The levels of aromatic amino acids decreased by 16% in leaves exposed to Cd.

Table 2 - Effects of Cd stress on nitrate reductase enzymatic activity in barley leaves and roots. Values represent an average measurement \pm SD. Asterisks indicate significantly different values in Cd stress vs control plants at $p \leq 0.005$ (*) and $p \leq 0.0005$ (**). Letter “a” show significant differences vs 7 days Cd exposed leaves. Letter “b” show significant differences vs 2 days exposed roots. Letter “c” show significant differences vs 7 days exposed roots.

Nitrate reductase activity (mmol min⁻¹ mg⁻¹)								
0h		1d		2d		7d		
Control	Cd	Control	Cd	Control	Cd	Control	Cd	
Leaves	0.0529 \pm 0.01	0.0531 \pm 0.02 a	0.0514 \pm 0.04	0.0726 \pm 0.03 ^{*a}	0.0522 \pm 0.02	0.103 \pm 0.06 ^{** a}	0.0521 \pm 0.01	0.123 \pm 0.01 ^{**}
Roots	0.170 \pm 0.01	0.168 \pm 0.003 ^{bc}	0.228 \pm 0.07	0.178 \pm 0.01 ^{bc}	0.272 \pm 0.002	0.800 \pm 0.06 ^{*c}	0.214 \pm 0.06	0.496 \pm 0.01 ^{** b}

Tables 3 - Amino acids concentration ($\mu\text{mol g}^{-1}$ FW) in leaves and roots of barley plants exposed to 10^{-5} M of Cadmium stress for 7 days in hydroponic culture. Values represent an average measurement \pm SD. Asterisks indicate significantly different values in Cd stress vs control plants at $p \leq 0.05$ (*) and $p \leq 0.005$ (**), respectively.

Leaves	Control ($\mu\text{mol g}^{-1}$ FW)	Cd ($\mu\text{mol g}^{-1}$ FW)	Roots	Control ($\mu\text{mol g}^{-1}$ FW)	Cd ($\mu\text{mol g}^{-1}$ FW)
<i>Asp</i>	1.57 \pm 0.090	1.25 \pm 0.032*	<i>Asp</i>	0.87 \pm 0.093	0.96 \pm 0.085*
<i>Glu</i>	0.36 \pm 0.016	0.49 \pm 0.01**	<i>Glu</i>	0.13 \pm 0.084	0.18 \pm 0.015
<i>Asn</i>	12.65 \pm 0.851	13.62 \pm 0.65*	<i>Asn</i>	4.23 \pm 0.154	4.04 \pm 0.384*
<i>Ser</i>	0.94 \pm 0.020	0.90 \pm 0.023	<i>Ser</i>	0.27 \pm 0.005	0.17 \pm 0.066*
<i>Gln</i>	1.23 \pm 0.608	0.71 \pm 0.042	<i>Gln</i>	2.35 \pm 0.072	2.13 \pm 0.187*
<i>His</i>	0.44 \pm 0.020	0.47 \pm 0.022	<i>His</i>	0.20 \pm 0.009	0.19 \pm 0.001
<i>Gly</i>	0.04 \pm 0.003	0.05 \pm 0.008	<i>Gly</i>	0.05 \pm 0.005	0.04 \pm 0.004**
<i>Thr</i>	0.45 \pm 0.026	0.42 \pm 0.018*	<i>Thr</i>	0.15 \pm 0.002	0.13 \pm 0.010*
<i>Arg</i>	0.41 \pm 0.024	0.34 \pm 0.02**	<i>Arg</i>	0.14 \pm 0.014	0.14 \pm 0.010
<i>Ala</i>	0.70 \pm 0.095	0.70 \pm 0.040	<i>Ala</i>	1.65 \pm 0.104	2.43 \pm 0.293*
<i>Tyr</i>	0.04 \pm 0.002	0.02 \pm 0.002*	<i>Tyr</i>	0.02 \pm 0.001	0.02 \pm 0.002
<i>Val</i>	0.27 \pm 0.101	0.16 \pm 0.009	<i>Val</i>	0.14 \pm 0.021	0.10 \pm 0.009*
<i>Trp</i>	0.36 \pm 0.148	0.31 \pm 0.114	<i>Trp</i>	0.41 \pm 0.074	0.57 \pm 0.097*

<i>Phe</i>	0.17 ± 0.025	0.15 ± 0.029*	<i>Phe</i>	0.19 ± 0.029	0.16 ± 0.010
<i>Ile</i>	0.12 ± 0.084	0.12 ± 0.050	<i>Ile</i>	0.10 ± 0.048	0.10 ± 0.052
<i>Leu</i>	0.14 ± 0.032	0.18 ± 0.061	<i>Leu</i>	0.12 ± 0.033	0.10 ± 0.031**
<i>Lys</i>	2.26 ± 0.253	1.92 ± 0.401*	<i>Lys</i>	1.53 ± 0.329	1.63 ± 0.389
<i>Cit</i>	0.03 ± 0.001	0.04 ± 0.002*	<i>Cit</i>	0.03 ± 0.001	0.03 ± 0.001*
<i>Orn</i>	0.29 ± 0.113	0.78 ± 0.102*	<i>Orn</i>	0.48 ± 0.146	0.44 ± 0.168*
Tot	22.47	22.63	Tot	13.099	13.59

DISCUSSION

Heavy metals (HMs) play important and ambivalent roles in plant metabolism: some of them can represent essential micronutrients for plant growth, but, when in excess, induce severe toxicity. These conditions are generally caused by industrial and on the other anthropic activities emitting toxic heavy metals such as cadmium, chromium, nickel and others (Parrota et al. 2015). The effects of heavy metal pollution depend on exposure time, quantity and by the specific element (Yousaf et al. 2016; Akhtar et al. 2017; Rizwan et al. 2017); among the heavy metals that contaminate the environment, cadmium causes great concerns, due to its solubility, which is able to cause serious problems to living organisms. This non-essential element is taken up from the plants through the roots and accumulates in many organs including roots, shoots, leaves and grains (Rizwan et al. 2017).

As described in this study, Cd exposition induced severe consequences in barley plants causing decrease in biomass, a reduction in leaf length, photosynthetic efficiency and a modification of the root system after Cd treatment. The reduction of these parameters is a common response of plants to heavy metals. Recently, an interesting correlated reduction upon increasing toxic concentration of Cd was reported in biomass and chlorophyll content in *Festuca arundinacea* (Huang et al. 2017). Furthermore, the correlation between RWC, pigments, dry weight and tissue length values were previously reported in various plants (barley, bean, *Brassica* and others) upon different heavy metals as Cd or Chromium (Gonzales et al. 2015; Nahar et al. 2016; Al Mahmud et al. 2017).

Similar results were previously shown in tomato plants exposed to cadmium and copper (Mediouni et al., 2006; Hediji et al. 2015); these morphogenetic changes are possibly not-specific symptoms of metabolic changes, generally observed under various stress such as drought (Landi et al. 2016). Furthermore, prolonged Cd stress conditions induced changes in several physiological and biochemical processes such as pigment content, osmoregulation by proline levels, and proteins preservation by HSP70s. It has been reported that different isoforms of HSP70s (chloroplastic, cytosolic and mitochondrial) play a critical role in pollution stress in different types of plant bio-

indicator Basile et al. 2015). Furthermore, alterations in photosynthetic machinery have been reported: it was worth pointing out that photosynthetic efficiency parameters (e.g Fv/Fm or QY) decreased upon heavy metals exposition (Arena et al. 2017).

Moreover, RuBisCO large subunit (LS), and D1 component of photosystem II showed an increase after 7 days of Cd exposition. Consistently with our results, both large and small RuBisCO subunits significantly increased upon Cd stress in spinach (*Spinacia oleracea* – Bagheri et al. 2017). Interestingly, heavy metals stresses showed different responses in RuBisCO large subunit (LS), and D1 in *Cynara cardunculus*: D1 proteins similarly increased upon Pb and Cd stress, while RuBisCO showed significant increases exclusively upon Cd exposure (Arena et al. 2017).

In this context, G6PDH, representing a major source of NADPH in the plant cell, would play a central role in plant stress response. Data presented in this work, together with previous results (Yang et al. 2014) suggest that a severe exposition to Cd induced an increase of G6PDH activities in leaves and roots. Thus, a central role in HM stresses response is proposed for this enzyme: in details, in leaves a first increase of G6PDH activity was reported after 6h of Cd exposition. The increase remained stable after 1 days and 2 days of Cd exposition, showing a crescent increase after 7 days. Similarly, our results suggest a major role for G6PDH in the first phase of Cd stress response in barley roots.

The enzymatic rate modification could also be associated to the abundance of G6PDHs isoforms observed in western blotting. It could be argued that G6PDHs increase is one of the main effects of heavy metals in response to the oxidative stress induced by pollution; therefore, the increase in the G6PDH activities appears as involved to preserve the basal cellular metabolism (Esposito et al. 2005). It is worth to point out that a major role for cy-G6PDH has already been demonstrated upon salt stress (Dal Santo et al. 2012; Cardi et al. 2015), ABA treatments (Cardi et al. 2011) and drought (Landi et al. 2016).

G6PDH represents one of the major sources of NADPH in the plant cell, and this is the main reductant required for the ascorbate-glutathione cycle (Letierrier et al. 2007; Esposito 2016). As

expected, APX activity increases under Cd stress; this increase of the APX activity in leaves plays a crucial role to counteract the increment of H₂O₂ as result of the oxidative damages induced by HM. APX and the other enzymes of the antioxidant systems (e.g. catalase, superoxide dismutase) act in first phase of the oxidative stress response (Van Oosten et al. 2016). Intriguingly, the G6PDH and APX correlation has been previously reported in various crops in order to reduce the negative effects induced by different stresses causing an oxidative burst (Gill and Tuteja, 2010).

The activity of G6PDH was also related to nitrogen metabolism, in this study monitored by nitrate reductase activity (Esposito, 2016). Plant exposed to Cd showed similar activities changes in both G6PDH and nitrate reductase. It has previously demonstrated that reducing power produced by G6PDH plays a critical role during nitrogen assimilation (Esposito et al. 2005). Furthermore, nitrate uptake and reduction play an emerging role in abiotic stress response, and nitrate reductase genes and nitrate transporters were recently identified as co-expressed with G6PDHs in *Arabidopsis thaliana* (Landi et al. 2017; Landi and Esposito 2017).

These metabolic changes induced by Cd exposition were compared with the free amino acids levels. Consistently, proline level increased upon Cd exposure in leaves, thus confirming that under stress, barley plants activate a complex range of responses in order to reduce the toxic effects and sustain growth under unfavourable conditions (Ashraf and Foolad 2007; Gill and Tuteja 2010; Landi et al. 2017). In response to the oxidative stress, plants activate different mechanisms, among these, proline accumulation in leaves plays a pivotal role (Ashraf and Foolad 2007).

Therefore, the nitrate assimilation increased by Cd could be related to the strong increase in proline levels possibly to counteract stress. In addition, it has been proposed that proline may represent an active H₂O₂, O₂ and OH⁻ scavenger (Gill and Tuteja 2010; Landi and Esposito 2017).

As expected, Cd induced changes in aminoacidic profile in both roots and leaves: plants exposed to metal toxicity accumulates specific amino acids which may play a role in the tolerance to stress and have beneficial functions as proline and others (Xu et al. 2012; Pavlíková et al. 2014a, b).

It is worth noting that proline levels (and isoleucine and valine as well) may regulate cell osmotic potential; on the other hand, methionine, glycine, cysteine, leucine play roles in plant cell growth and cell wall organization; furthermore, levels of aromatic amino acids could modulate protein synthesis and production (Joshi et al. 2010; Pavlíková et al. 2014a; Zemanova et al. 2017).

In the roots, the increase in aspartate content corresponds to decrease in asparagine and glutamine levels, possibly limiting the transport of these amino acids to the aerial part of the plant. The accumulation of alanine in the roots indicated an overflow of pyruvate coming from increased root glycolysis induced by stress.

Barley leaves showed an opposite behaviour compared to roots in asparagine accumulation, suggesting different nitrogen re-utilization and accumulation mechanisms. Barley plants exposed to Cd showed increased levels in phenylalanine, tryptophan and tyrosine. Intriguingly, the pathways involved in aromatic aminocid synthesis (as Shikimate cycle), are strictly connected with G6PDH activity, which provides the NADPH required for these processes. Therefore, in the leaves the reported reduction of phenylalanine, tryptophan and tyrosine levels would indicate a diversion in NADPH usage to antioxidant stress response.

CONCLUSION

The data here presented suggest a primary role of G6PDH in the response to cadmium stress in barley. These results corroborated the idea that the abiotic stress response in plants involves G6PDH; this activity may play a pivotal role in assisting various physiological aspects of the stress, giving its central role played in ROS scavenging, nitrogen assimilation and basal metabolic processes.

Further studies are required to elucidate both the complete pathway(s) of signalling, from detection of stress to the increase in G6PDH activity, both the mechanisms providing a possible feedback modulation of delivery of reductants by this pivotal enzyme of cell metabolism.

AUTHOR CONTRIBUTIONS STATEMENT

M.L. conceived and made the experiments, first author; A.D.L. made enzymatic experiments and grew plants joint first author; V.P., made WB and wrote the paper; D.L. grew plants and made WB; S.L. measured enzymatic activities, wrote and amended the paper; S.E. conceived the work and the experiments; wrote and amended the paper, corresponding author.

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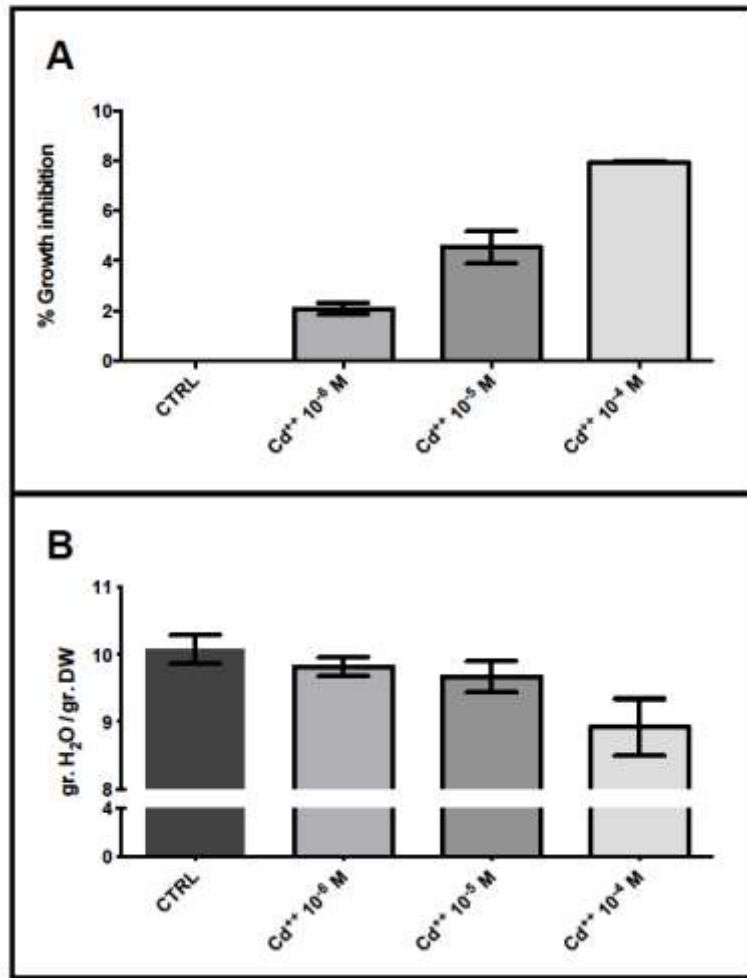
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Supplemental Figure 1 – (A) Percentage of inhibition of growth; and (B) water content in young barley plants in hydroculture exposed for 7d to different cadmium concentrations. Other details in Materials and Methods.

General Conclusions

Among the many stress that mostly affect crops a prominent role is played by metalloids and heavy metal contaminations.

A number of key genes control or contribute to the plant response to toxic concentrations of metalloids. These genetic traits regulate molecular and physiological mechanisms for a genetic improvement of crops. In addition to model plants as *A. thaliana*, novel insights have been provided by using crop models, especially rice (*Oryza sativa*), in order to find new processes for effective crop-field applications. In the last decades, new genomes and transcriptomes are emerging daily for new species, but a thorough functional characterization is still necessary.

Heavy metal toxicity is another increasing environmental threats to the ecosystems, caused by both natural sources and human activities. Among the many metals, Zn represents one of the most widespread pollutants. Two horticultural plants exhibiting different tolerance to Zn, *Lactuca sativa* cv. Phillipus, and *Brassica oleracea* cv. Bronco, exposed to Zn excess, demonstrated the contribution of compatible osmolytes as proline, glycine betaine and γ -aminobutyric acid in the mechanism(s) that plants act to face Zn stress. Another study carried out with cadmium, one of the most toxic pollutants in plant ecosystems, showed that this metal at high concentrations can cause severe effects, such as plant growth inhibition, decrease in photosynthesis and changes in plant basal metabolism. The presented data suggest that G6PDH may play a pivotal role to counteract the oxidative stress induced by cadmium in young barley plants, affecting ROS scavenging, nitrogen assimilation and basal metabolic processes.

Si nutrition was already extensively shown to be important in the protection against various abiotic stresses, and the use of this element against toxic concentrations of metalloids and heavy metals could provide additional sustainable agricultural practices.

The data presented in this thesis, obtained in a study carried out with *Hordeum vulgare* exposed to Zn deficiency and toxicity conditions, clearly demonstrated the extensive stress induced by not-optimal Zn nutrition and the generic beneficial effects of the silicate in ameliorate oxidative stress,

photosynthetic machinery and affect nitrogen and carboxylate metabolism. Particularly, a diversion of nitrogen metabolism and a reduction of photorespiratory metabolism induced by Si supply are able to counteract the effect of Zn limitation or excess, thus improving the overall crop growth.

Lista delle Pubblicazioni della Dott.^{ssa} Valeria Paradisone

Pubblicazioni Indicizzate ISI-WoS

1. Paradisone V, Barrameda Medina Y, Montesinos Pereira D, Romero L, Esposito S, Ruiz JM (2015) Roles of some nitrogenous compounds protectors in the resistance to zinc toxicity in *Lactuca sativa* cv. Phillipus, and *Brassica oleracea* cv. Bronco. *Acta Physiologiae Plantarum*. 37: 137 DOI: 10.1007/s11738-015-1893-9
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1. Landi S., Paradisone V., Esposito S. (2019) Molecular investigation on metalloids stress response in higher plants: recent insight from toxicity to tolerance in ““Role of Metalloids in Plants: Advances and Future Prospects” R. Deshmukh, D. K. Tripathi, G. Guerriero (Eds). Wiley 2019
2. Paradisone V., Landi S., Esposito S. (2019) Roles of Silicon in alleviating Zinc stress in plants in ““Role of Metalloids in Plants: Advances and Future Prospects” R. Deshmukh, D. K. Tripathi, G. Guerriero (Eds). Wiley 2019

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1. De Lillo A., Lutterbey M.-C., Lansing H., von Schaewen A., Paradisone V., Esposito S. (2016) Subcellular localization analyses of a *Hordeum vulgare* P2-G6PDH isoform by transient expression of reporter fusion proteins. Communication at FISV 2016 - XIV Congress of the Italian Federation of Life Sciences (FISV) - Rome - September 20th – 23rd 2016.
2. Lentini M., Sorbo S., De Lillo A., Paradisone V., Arena C., Basile A., Esposito S. (2016) Ultrastructural and physiological changes in barley seedlings exposed to cadmium - Communication at FISV 2016 - XIV Congress of the Italian Federation of Life Sciences (FISV) - Rome - September 20th – 23rd 2016.
3. Paradisone V., Lentini M., De Lillo A., Esposito S. (2016) Effect of zinc deficiency in *Hordeum vulgare*: are changes in morphological and physiological aspects related to alterations in nitrate uptake? - Communication at FISV 2016 - XIV Congress of the Italian Federation of Life Sciences (FISV) - Rome - September 20th – 23rd 2016

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5. Paradisone V., Navarro Leon E., Blasco B. , Ruiz J.M., Esposito S. (2017) Silicon ameliorates Zn deficiency symptoms in relation to photosynthesis and oxidative stress. Poster at Joint Congress SIBV-SIGA "Sustainability of agricultural environment: contribution of plant genetics and physiology " PISA, 19th-22nd September, 2017
6. Paradisone V., Landi S., Esposito S. (2018) Damages induced by Zn limitation and excess can be relieved by Silicon in barley. Poster at Plant Biology Europe FESPB/EPSO Congress, Copenhagen, Denmark, 18-21 June 2018
7. Paradisone V., Navarro-Leon E., Esposito S., Ruiz J.M., Blasco B. (2018) Effect of Silicon on amino acids profile in Zinc-deficient Barley plants. Poster at Nutriplanta - XVII Simpósio Luso-Espanhol de Nutrição Mineral das Plantas Lisbon, July 25th – 27th, 2018
8. Landi S., Capasso G., Paradisone V., Hammami Z., Ezzahra Ben Azaiez F., Ayadi S., Esposito S. 2018 Mediterranean barley (*Hordeum vulgare*) genotypes as a tool to identify specific roles for HSP70s in response to abiotic stress - Communication at FISV 2018 - XV Congress of the Italian Federation of Life Sciences (FISV) - Rome - September 18th – 21st 2018