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Facultad de Ingeniería, Ciencias y Administración
Programa de Doctorado en Ciencias de Recursos Naturales

MANGANESE (Mn) EXCESS IN BLUEBERRY CULTIVARS (*Vaccinium corymbosum* L.): POSSIBLE MECHANISMS OF TOXICITY AND RESISTANCE

TESIS PARA OPTAR AL GRADO ACADÉMICO
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RAYEN XIMENA MILLALEO MILLALEO

TEMUCO – CHILE
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Thesis outline

Acid soils, such as Ultisol and Andisol of southern Chile, have volcanic ash origin. The main characteristics of these soils are the high organic matter (OM) content, low availability of essential elements for plants such as nitrogen (N), phosphorus (P), calcium (Ca), magnesium (Mg) and micronutrients (e.g. molybdenum, Mo), and pH values lower than 5.5. This last characteristic is associated with high available contents of aluminum (Al) and manganese (Mn), which can be toxic for crops reducing their productivity. Mn is influenced by soil redox conditions, where the reduced form of this metal, Mn^{2+} is the available form for plants.

Manganese is an essential micronutrient for plants. In plant cells, it participates in various metabolic cell processes, such as photosynthetic pathways, enzyme activation, and diverse metabolites syntheses. Nevertheless, Mn excess in acid soils causes various alterations at physiological and biochemical level in plants according to its toxicity. Thus, these toxic effects in plants alter the photosynthetic capacity, estimulating the reactive oxygen species (ROS). ROS are highly harmful to biological membranes, which can cause alterations including to the cell death. To counteract this damage, plant cells have developed several mechanisms that remove ROS.

In Chile, highbush blueberry (*Vaccinium corymbosum* L.) is grown in the southern regions where is well adapted to acid soils (pH ranges from 4.0 to 5.5), with high levels of organic matter (OM), even in flooded conditions. Fruits are a good source of natural antioxidants, considered beneficial for human health.

The general aim of this thesis is to evaluate the Mn toxicity effect on physiological and biochemical properties of photosynthetic apparatus, as well as to analyze its resistance mechanisms to Mn excess in highbush blueberry cultivars. Additionally, the effect of Mn excess on photosynthetic performance in *Arabidopsis thaliana* as a model plant under Mn excess was studied.

In the first Chapter a General Introduction is presented, where an overview about the Mn excess in plants is showed. In Chapter 2, an overview on Mn transport, accumulation and resistance mechanism are discussed.

In Chapter 3 biochemical and physiological differential responses of three cultivars of blueberry (Legacy, Brigitta and Bluegold) subjected to increasing Mn concentrations in nutrient solutions were analyzed. The results showed no evidence of visual symptoms, but significant differences in biomass and photosynthetic parameters were found. Significant reductions in aerial biomass (33%) in Bluegold with the highest Mn treatment (1000 µM) compared with the control were observed. Brigitta and Legacy showed the best growth of the aerial biomass remaining stable under Mn treatments. In contrast, root growth was not significantly different in all cultivars subjected to Mn excess. Moreover, some mechanisms of tolerance as the antioxidant system and the exudation of organic compounds indicated less damage in Legacy, where radical scavenging activity (RSA) values had no significant changes in leaves. However, Bluegold exhibited the higher RSA values in leaves with the highest Mn treatment. With respect to organic acid anions root exudates, oxalate, and citrate presented the greater values in Legacy and Brigitta.

In Chapter 4, we reported that an excess of Mn in plants has a negative effect on the photosynthetic performance, especially in the first light reactions involving the photosystems I and II (PSI and PSII). The aim of this research was evaluate the role of specific Mn-induced changes in the structure and function of PSII and PSI, to help to understand the mechanisms by which Mn excess may cause a decrease in CO_2 assimilation in *Arabidopsis thaliana.* We analyzed PSII and PSI operation, together with the protein composition of the photosynthetic apparatus against increasing levels of Mn. In agreement with earlier studies Mn excess caused minimal changes in the maximal photochemical efficiency of PSII measured as Fv/Fm, although the characteristic peak temperature of the $S_{2/3}Q_B$- charge recombinations was shifted to lower temperatures at the highest Mn concentration. SDS-PAGE and immunoblot analyses also did not exhibit any significant change in the relative abundance of PSII-associated polypeptides: PSII reaction center protein D1, Lhcb1 (major light harvesting protein of LHCII complex) and PsbO (OEC33 - 33kDa protein of the oxygen-evolving complex). In addition, the abundance of Rubisco did

not change with Mn treatments. Nonetheless, plants grown under excess Mn exhibited increased susceptibility to PSII photoinhibition. In contrast, *in vivo* measurements of the redox transients of PSI reaction centre (P700) showed a considerable gradual decrease in the extent of P700 photooxidation ($P700^{+}$) under increased Mn concentrations compared to control. This was accompanied by a slower rate of $P700^{+}$ re-reduction indicating a down-regulation of the PSI-dependent cyclic electron flow. The abundance of PSI reaction center polypeptides (PsaA and PsaB) in plants under the highest Mn concentration was also significantly lower compared to the control. The results demonstrate for the first time that PSI is the major target of Mn toxicity within the photosynthetic apparatus of *Arabidopsis* plants. The possible involvement mechanisms of Mn toxicity targeting specifically PSI are discussed.

Finally, in Chapter 5 and 6 we present the general discussion and conclusions of the thesis, concluding that a Mn excess (1000 μM Mn) has frequently negative effects on physiological and biochemical parameters of the essayed blueberry cultivars. The most noticeable negative changes were exhibited by Bluegold, where the photosynthetic performance and aerial biomass were more reduced than in Legacy and Brigitta.

Table of contents

Table Index

Figure Index

Chapter 1

General Introduction

1.1 Introduction

1.1.1 Acid soils

Acid soils represents up to 50% worldwide arable soils. Moreover, tropical and subtropical zones have a special significance because around 60% of their soils are acids, limiting the crops growth in several developing countries (Kochian et al., 2004). Acidification can be caused by high pluviometry in winter, which leads to a loss of interchangeable bases (Mora, 1993; Mora et al., 2006), high use of fertilizers acid reaction (ammonium type), with consequent decrease of calcium (Ca), magnesium (Mg), phosphorus (P) and/or molybdenum (Mo) availability (Foy et al., 1978; Mora et al., 1999).

Acid soils are found in southern Chile, with Andisol and Ultisol types mainly, from volcanic origin. These soils are developed under a cold and wet climate, with high organic matter content, and low availability of P and nitrogen (N) (Aguilera et al., 1997, 2002; Peñaloza et al., 2004). The main constraint reported in acid soils by pH among 4 to 5.5, is an increase of metals cation solubility, as aluminum (Al) and manganese (Mn) (Jarvis, 1987; Watmough et al., 2007), being Mn the second phytotoxic element after Al which limits growth of plants (Foy, 1984; Kochian et al. 2004).

1.1.2 Mn in soils.

Apart of low pH, redox changes conditions of soils determine the Mn availability, solubility (Marschner, 1995; Pittman, 2005; Sposito, 1989; Gherardi and Rengel, 2004) and Mn content (Farasova and Beinrohr, 1998). According, Boojar and Goodarzi (2008), low pH enhance the Mn solubility to accelerate its release from soil solution. Under reduction conditions, soils have major amount of free electrons, which may be increased by water excess, poor drainage, waterlogged places or heavy organic matter applications, among others, even in soil with high pH (Sparrow and Uren 1987; Hue, 1988). In addition, to remove electron acceptor agents (as oxygen) from surrounding soil, there are an increase in the Mn availability (Porter et al., 2004). Therefore, Mn phytoxicity is favored in acid and/or waterlogged soils, where both pH (<5.5) and redox conditions increase the concentration of the soluble Mn (Mn^{2+}), which is the predominant Mn form in the soil acid solution (Marschner 1995; Porter et al. 2004).

1.1.3 Mn as micronutrient in plants.

Mn is considered an essential micronutrient to plant growth and development. It can be absorbed mainly as Mn^{2+} ions (Mukhopadhyay y Sharma, 1991). Specifically, it is involved in water splitting in the photosystem II (PSII) of the photosynthetic process (Goussias et al., 2002). Also, Mn is a constitutive element associated with the oxygen evolving complex (OEC) of photosystem II (PSII), an important multi-protein pigment complex embedded in the thylakoid membranes (Hankamer et al., 1997; Enami et al., 2008). Therefore, the Mn cluster, together with other ions and extrinsic proteins that constitute OEC are required to oxidize water and reduce P680, the reaction center of PSII (Kern and Renger, 2007; Ferreira et al., 2004; Rutherford and Boussac, 2004). In conjunction with photosystem I (PSI) and linear electron transport, these reducing equivalents (electrons) are used primarily in the conversion of CO_2 into carbohydrate (Ferreira et al., 2004). Mn also participates as metallic cofactor in superoxide dismutase enzyme (MnSOD), located in mitochondria of eukaryotic organisms and into SOD iso-enzymes group such as Fe (FeSOD), copper and zinc enzymes (Cu/ZnSOD) (Blokhina et al., 2003).

It has been estimated that Mn normal values of various agricultural crops range from 30 to 500 mg kg^{-1} dry weight (DW) (Humpries et al., 2007). Pasture species had values fluctuating between 5 to 1200 mg kg^{-1} in different zones (Whitehead, 2000). In southern Chile, species as white clover (*Trifolium repens* L.) and perennial ryegrass (*Lolium perenne* L.) have values of Mn concentrations between 50 to 1000 mg kg^{-1}. Into bush species as blueberries (*Vaccinium corymbosum* L.), values up to 1600 mg kg^{-1} in autumn and values between 280 to 560 mg kg^{-1} of Mn are reported (Universidad de La Frontera, Soil and Plant Analysis Laboratory Services).

1.1.4 Physiological and biochemical effects of Mn excess in plants.

As mentioned, trace element Mn can be a toxic element in plants when it is in excess (Marschner, 1995). Mn toxicity can show visuals symptoms as chlorosis, brown speckles in the leaves (Wissemeier and Horst, 1992; González et al., 1998). Besides, Mn excess can produce several detrimental effects such as growth reduction (Mora et al., 2009), modifying

various processes such as activity of enzymes, absorption, translocation and utilization of other mineral elements (Ca, Mg, Fe and P) (Ducic and Polle, 2005; Lei et al 2007).

The Mn excess seems also to be particularly damaging to physiological processes as well as the photosynthetic apparatus (Mukhopadhyay and Sharma, 1991). In this context, it has been demonstrated that high Mn accumulation in thylakoids it is associated with inhibition of the net photosynthesis and carboxylation efficiency, suggesting that this metal may interfere with the energy fluxes by thylakoid stacking (Lidon and Teixeira, 2000; Lidon et al., 2004). The decline of photosynthesis is one of the mechanisms constituting the toxic effects of Mn excess, being an early indicator for Mn toxicity in tobacco (Nable et al., 1988). Furthermore, an inhibition of photosynthesis and photochemical parameters of Cucumber leaves was observed by increasing Mn concentrations, producing stomatal closure with a lower accumulation of biomass (Feng et al., 2009).

1.1.5 Mechanism against Mn excess.

To counteract and ameliorate the detrimental effects of Mn excess, plant tissues have Mn resistance mechanisms that can include avoidance or exclusion (Delhaize et al., 2007a) and tolerance or inclusion. The first one involves a protective role that prevents that the metal ions entering the cytoplasm of plant cells (Blamey et al 1986; Marschner 1991) and the second one (tolerance or inclusion) implies a detoxification of metal ions after they have crossed the plasma cell membrane or internal biomembranes of organelles (Taylor, 1991). Thus, organic acids exudations can be considered as resistance mechanisms against metal toxicity in several plant species (Delhaize et al., 2007a; Mora et al., 2009; Ryan et al., 2009). Besides, in case of Mn deficiency, some organic acids act as metal chelators in the rhizosphere, increasing the availability of metallic soil micronutrients (Dakora and Phillips, 2002).

Otherwise, one research area that have relevance to the field of Mn tolerance mechanisms in plant cell, involves the identification of several and numerous plant genes that encode transporters with the ability to internal transport, sequestrations, distribution and

homeostasis of Mn^{2+} (Pittman, 2005; Kochian et al 2004; Delhaize et al., 2007b; Sasaki et al., 2011; Hirschi et al., 2000). In the case of Mn, researches about genes expression suggested that genes associated to internal Mn tolerance are more abundant than those involved with Mn exclusion (Horst, 1988; Delhaize et al., 2003).

Other mechanism that can withstand an abiotic stress (as metal toxicity) is the activation of antioxidant systems. Reactive oxygen species (ROS) are intermediates products of molecular oxygen reduction to water molecules which cause damage at cell level (Schützendübel and Polle, 2002). Under normal conditions, plant cell generate ROS, which may increase when plant organelles are under both biotic and abiotic environmental stresses (Fecht-Christoffers et al., 2003). Effects of ROS involve oxidative damages on cell biomolecules such as lipids, proteins and nucleic acids, leading to a biomembrane peroxidation, loss fluidity, protein hydrolysis, inactivation of enzyme and even DNA chain breakdown (Demirevska-Kepova et al., 2004; Guo et al., 2007; Hegedüs et al., 2001; Feng et al., 2009; Srivastava and Dubey, 2011).

Antioxidant defense mechanisms can prevent and diminish the detrimental effects of ROS. These systems involved enzymatic mechanisms, as superoxide dismutase (SOD), peroxidase (APX), catalase (CAT), glutation peroxidase (GPX) and non-enzimatic mechanisms as ascorbate, α-tocopherol, alkaloids, carotenoids, flavonoids and glutation (Foyer and Noctor, 2003; Apel and Hirt, 2004). Studies on Mn excess showed significant changes in antioxidant systems of some plants. Specifically, SOD activity is reported as a Mn-tolerance mechanism in rye grass cultivars subjected to Mn excess (Mora et al. 2009). Rosas et al., (2007) reported high activity of APX values in Mn toxic treatments (355 µM Mn) in prairie species, indicating an oxidative stress. In the same way, several of these enzymes are considered scavengers of ROS produced by Mn toxicity in rice and cucumber plants (Srivastava and Dubey, 2011; Feng et al., 2009). In addition, Mn is required as a cofactor for Mn-SOD enzyme, essential to the protection against oxidative stress in plants (Burnell, 1988; Bowler et al., 1994).

1.1.6 Blueberry crop in Chile.

In Chile, *Vaccinium corymbosum* L. (highbush blueberry) is an important crop, whith 12,000 ha, concentrated in the South Central area. Recently, in the region where our study was carried out, there are approximately 1,500 ha of blueberry crop (ODEPA and CIREN, 2012). Likewise, its production reached 100,000 ton approximately in year 2011, being Chilean blueberry industry a global leadership and the largest exporter of this species (ODEPA, 2012).

Blueberry fruits are considered the richest sources of antioxidant compounds of different species of fresh fruits and vegetables which contribute to a high antioxidant capacity (Prior et al., 1998; Howard et al., 2003). Phenolic acids, anthocyanins and flavonoids are the phytochemicals that mostly influence the antioxidant capacity of plants (Prior et al., 1998).

Although, blueberry crop is well adapted to soils under acidic conditions (Ireland and Wilk, 2006), the effect of Mn excess on this crop is unknown. Therefore, the main focus of this investigation intends to clarify the physiological and biochemical response of *V. corymbosum* under excess of Mn, in order to elucidate the tolerance mechanisms involved in the Mn excess. In addition, a complementary study was conducted to obtain information concerning to the toxic effect(s) of excess of Mn on the polypeptides of both PSII and especially PSI using the model plant *Arabidopsis thaliana.*

1.2 Hypotheses of this thesis

High concentrations of available Mn (Mn^{2+}) are associated with soil acidity.These ions can be accumulated in plant cell tissues producing toxicity, which alters physiological and biochemical features. This can stimulate defense plant mechanisms to exclude or tolerate this toxicity.

We hypothesized:

i) Blueberry cultivars will have differential capacities to accumulate Mn and therefore different capacity to tolerate oxidative stress.

ii) The Mn-resistant cultivar will have a higher photosynthetic capacity and better Mn-tolerance mechanisms. Also, in a model plant, Mn excess decreases the activity and/or performance of the photosystems (PSI and PSII) involved in the photosynthetical apparatus.

1.3 General aim

To evaluate Mn toxicity effects on physiological and biochemical properties of photosynthetic apparatus in highbush blueberry cultivars, as well as to analyze their resistance mechanisms by Mn excess. Adittionally, to evaluate the susceptibility of the proteins of PSI and PSII to Mn excess in a model plant (*Arabidopsis thaliana*).

1.4 Specific aims

1. To evaluate specific mechanisms of Mn resistance (inclusion and/or exclusion) and antioxidant mechanisms in a Mn tolerant and a Mn sensitive cultivar of blueberry in the presence of Mn excess.

2. To compare physiological changes of photosynthetic apparatus in a Mn tolerant and a Mn sensitive blueberry cultivar under Mn excess.

3. To evaluate changes in proteins related to photosystems (PSI and PSII) in a plant model exposed to an excess of Mn.

Chapter 2

Theoretical background.

Manganese as essential and toxic element for plants: transport, accumulation and resistance mechanisms.

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Manganese as essential and toxic element for plants: transport, accumulation and resistance mechanisms

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Abstract

Manganese is an essential element for plants, intervening in several metabolic processes, mainly in photosynthesis and as an enzyme antioxidant-cofactor. Nevertheless, an excess of this micronutrient is toxic for plants. Mn phytotoxicity is manifested in a reduction of biomass and photosynthesis, and biochemical disorders such as oxidative stress. Some studies on Mn toxicity and Mn translocation from soil to plant cells in Mn^{2+} form have demonstrated their importance under low pH and redox potential conditions in the soil. When Mn is inside the cells, mechanisms that can tolerate this toxicity are also observed, being important the compartmentalization of this metal in different organelles of shoot and leaf plant cells. A key role of antioxidative systems in plants in relation to high Mn amounts has also been reported as a defense mechanism. The purpose of this review is to show the role of Mn as an essential micronutrient and as a toxic element to higher plants as well as to their transport and tolerance mechanisms. The forms and dynamics of this element in soils and the importance of the acidity for this dynamic and availability for plants are also given.

Keywords: Manganese, Mn toxicity, resistance mechanisms.

2.1 Introduction

Manganese (Mn) is an essential micronutrient in most organisms. In plants, it participates in the structure of photosynthetic proteins and enzymes. Its deficit is dangerous for chloroplasts because it affects the water-splitting system of photosystem II (PSII), which provides the necessary electrons for photosynthesis (Buchanan, 2000). However, its excess seems also to be particularly damaging to the photosynthetic apparatus (Mukhopadhyay and Sharma, 1991). Thus, Mn has two roles in the plant metabolic processes: as an essential micronutrient and as a toxic element when it is in excess (Kochian et al., 2004; Ducic and Polle, 2005). Mn toxicity is favored in acid soils (Pendias and Pendias, 1992). With decreasing pH, the amount of exchangeable manganese – mainly Mn^{2+} form – increases in the soil solution. This Mn form is available for plants and can be readily transported into the root cells and translocated to the shoots, where it is finally accumulated (Marschner, 1995). In contrast, other forms of Mn predominate at higher pH values, such as Mn (III) and Mn (IV), which are not available and cannot be accumulated in plants (Rengel, 2000).

Excessive Mn concentrations in plant tissues can alter various processes, such as enzyme activity, absorption, translocation and utilization of other mineral elements (Ca, Mg, Fe and P), causing oxidative stress (Ducic and Polle, 2005; Lei *et al*., 2007). The threshold of Mn injury as well as the tolerance to an excess of this metal is highly dependent on the plant species and cultivars or genotypes within a species (Foy *et al*., 1988, Horst, 1988).

The purpose of this review is to illustrate the most current understanding about Mn role as an essential micronutrient and as a toxic element to higher plants, the long distance and cellular transport in plants as well as the mechanisms or strategies involved for to resist an overload of this metal. The forms and dynamics of this element in soils and the importance of the acidity for this dynamic and availability to plants are also given.

2.2 Manganese forms and dynamics in soils.

Manganese biogeochemistry in soils is complex, because it is present in several oxidation states (0, II, III, IV, VI and VII), while in biological systems it occurs preferably as II, III and IV. Divalent manganese (Mn II) is the most soluble species of Mn in soil, whereas the solubility of Mn III and Mn IV are very low (Guest *et al*., 2002). Mn oxides can form co-

precipitates with iron (Fe) oxides, exhibiting amphoteric behavior. In addition, Mn interacts both with cations and anions in oxidation-reduction reactions involving Mn. These reactions are influenced by a variety of physical, chemical and microbiological processes (Bradl, 2004).

Both pH and redox conditions influence Mn bioavailability in soils (Marschner, 1995; Porter *et al*., 2004). In most acid soils at low pH (<5.5) and an increased redox potential of Mn, oxides can be easily reduced in the soil exchange sites (Kogelmann and Sharpe, 2006), increasing the concentration of soluble Mn^{2+} (Watmough *et al*., 2007), which is the predominant Mn form in the soil solution (Adriano, 2001) and the most available Mn form for plants (Marschner, 1995). At higher soil pH (up to pH 8), chemical Mn^{2+} auto-oxidation is favored over MnO_2, Mn_2O_3, Mn_3O_4 and even Mn_2O_7, which are not normally available to plants (Ducic and Polle, 2005; Humpries *et al*., 2007; Gherardi and Rengel, 2004). Furthermore, high pH allows Mn adsorption into soil particles, decreasing their availability (Fageria *et al*., 2002). Nevertheless, some reports have suggested that an excess of available Mn is produced under reduced soil conditions, even at high soil pH values (Hue, 1988). A reducing environment can be produced when there is an excess of water, poor drainage or applications of organic material (Hue, 1988; El-Jaoual and Cox, 1998). Different organic molecules can dissolve solid Mn oxides through transfer of electrons, transforming them into an available Mn form for plants (Laha and Luthy, 1990). Soil acidification is also accentuated by abundant pluviometry during winter, causing the main cations to leak from the soil (Mora *et al*., 2006). On the other hand, lime application is a key factor in decreasing soluble Mn in acid soils with a high Mn content, given that it can increase soil pH (Hue and Mai, 2002). The total Mn content in soils is variable. Sparks (1995) reported small amounts of Mn in soils, fluctuating from 20 to 10,000 mg kg^{-1} soil, whereas other authors have registered total Mn contents between 450 and ~ 4,000 mg Mn kg^{-1} soil (Adriano, 2001). In addition, the total Mn soil content was from 15 to 17 mg kg^{-1} in acid soils without liming (pH about 4.4) (Hue and Mai, 2002). In liming acid soils, the interchangeable Mn concentration varied from 14 to 96 mg kg^{-1} soil in one year, with higher concentrations under high moisture and temperature conditions (Conyers *et al*., 1997). In Chilean volcanic soils, so-called Andisols, Mn concentrations fluctuate between 4.5 and 80 mg kg^{-1} depending on the agronomic management. Moreover, the Mn amount is

higher in pasture soils (up to 400 mg kg^{-1}) mainly in winter (Data from Laboratorio de Análisis de Suelo y Planta, Universidad de La Frontera, Temuco, Chile). Environmental conditions also affect Mn soil contents. The highest concentrations of soluble and exchangeable Mn are found after hot, dry summers and under warm waterlogged conditions in acid soils. This is probably due to the inhibition of Mn-oxidizing organisms, thereby allowing the chemical reduction of Mn oxides in these soils (Sparrow and Uren, 1987; Conyers *et al*., 1997).

2.2.1 Manganese dynamics in the rizosphere

Rhizosphere, which is the narrow zone of soil immediately surrounding the root system, is of great importance for mineral plant nutrition. In this zone, both the mobilization and immobilization of nutrients occur (Marschner, 1995). As shown in Figure 2.1, a mobilization of Mn^{2+} is produced by the rhizosphere acidification due to the release of H^+ or low molecular weight organic acids (LMWOA) from plants (Rengel and Marschner, 2005). Organic acids released in anion forms from roots can chelate Mn^{2+} released from the MnOx (Mn oxides) (Ryan *et al.,* 2001). Neumann and Römheld (2001) reported that mobilization of micronutrients (including Mn) into the rhizosphere is due mainly by its acidification and complexation with the organic acids (citrate) in various plant species. It has been reported that organic amendments (chip compost and pine bark) applied to melon plants released organic compounds such as arabinose and malic acid that can dissolve MnO_x (Tsuji *et al*., 2006). Soil microorganisms can also help Mn mobilization and immobilization, depending on soil conditions (Marschner, 1995). In aerated soils, microorganisms may mobilize Mn through MnOx reduction favored by H^+ root excretion. In contrast, Mn-oxidizing bacteria can decrease Mn availability in aerated and calcareous soils or in poorly aerated and/or submerged soils. Another key factor in the Mn dynamics in soil is organic matter (OM). Given that OM is negatively charged, it has a great Mn adsorption capacity, forming Mn complexes which decrease the amount of exchangeable Mn. However, the Mn adsorbed by OM can be exchanged by the H^+ released from the roots (Bradl, 2004).

2.3 Manganese transport and accumulation in plants

As mentioned above, reduced Mn (Mn^{2+}) form is the only available metal form for plants. It can be taken up via an active transport system in epidermal root cells and transported as divalent cation Mn^{2+} into the plants (Marschner, 1995; Gherardi and Rengel, 2003; Pittman, 2005). Manganese uptake by roots is characterized as a biphasic process. The initial and rapid uptake phase is reversible and non-metabolic, with $Mn2^{+}$ and $Ca2^{+}$ or other cations being freely exchanged in the rhizosphere. In this phase, Mn^{2+} appears to be adsorbed by the negatively charged cell wall constituents of the root-cell apoplastic spaces (Humphries *et al*., 2007; Clarkson, 1988). The second phase is slow, with Mn^{2+} being less readily exchanged. Its uptake into the symplast is dependent on plant metabolism (Maas and Moore, 1968), although the exact mechanisms are not clear (Humphries *et al*., 2007). It has been shown that in transgenic tobacco transformed with a tomato root protein with a metal binding side at its N-terminus (LeGlp1), Mn binds to this protein. This strongly suggests the involvement of LeGlp1 in Mn uptake from the soil (Takahashi and Sugiura, 2001). Kinetic measurements have demonstrated 100 to 1,000 times higher rates of Mn transport than the estimated plant requirement for this element (Clarkson, 1988). These transport rates are explained by the high capacity of ion carriers and channels in the Mn ion transportation through the plasma membrane at a speed of several hundred to several million ions per second per protein molecule (Humphries *et al.*, 2007). According to these authors, Mn distribution from root cells within the whole plant involves primary transport in the xylem, transference from the xylem to the phloem and re-translocation into the phloem. Xylem transport from roots to the above-ground parts of plants is performed by the transpiration stream, whereas phloem transport is more selective, taking place from sources to sinks (Marschner, 1995).

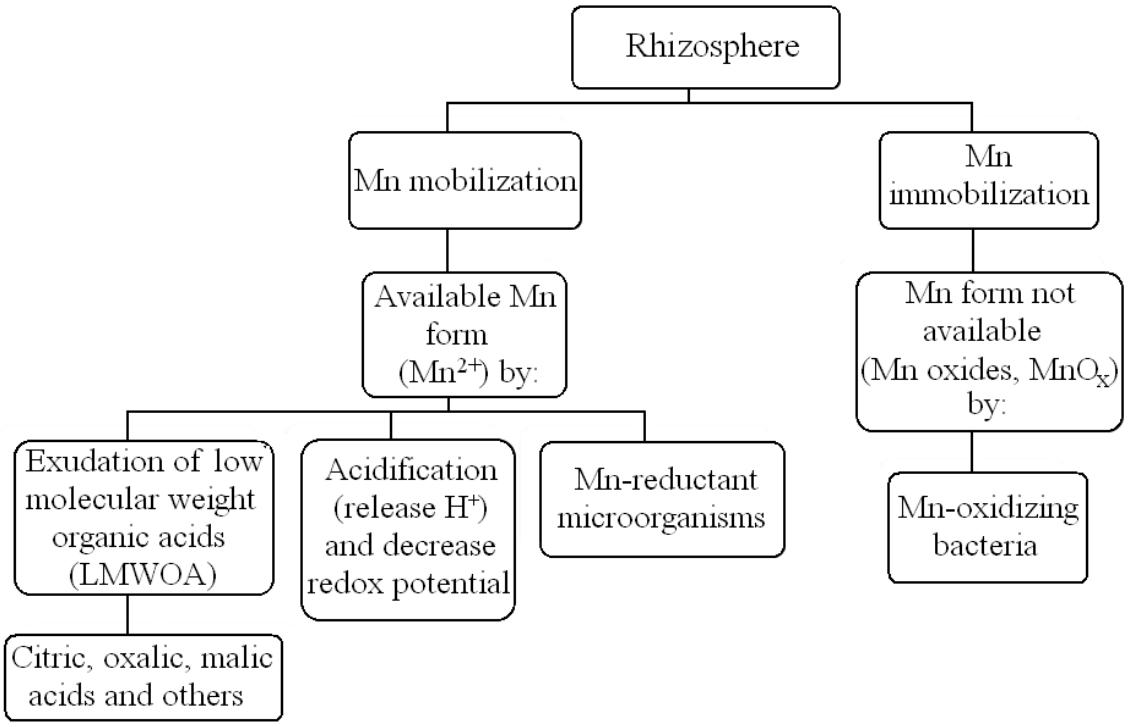

Figure 2.1. The role of soil rhizosphere in the mobilization and immobilization of Mn in soils.

Nonetheless, a low mobility in phloem has been reported for Mn, and its redistribution may depend on the plant species and stages of development (Herren and Feller, 1994). In fact, it has been reported that Mn transport from roots to grains is frequently insufficient at a mature stage of wheat. The relatively poor Mn mobility in the phloem emphasizes the importance of xylem in the transport of this element, even in wheat grain discharge (Rengel, 2001). Manganese generally tends to accumulate predominantly in the plant shoots than in the roots, as demonstrated in Mn labeling experiments with ^{54}Mn at an early stage of wheat (*Triticum aestivum* cv. Arina) development, where a fast Mn transport from roots to shoots was visualized in the xylem and was essentially immobile in the phloem (Page and Feller, 2005). Similar effects on Mn translocation have been shown by the same technique in young (28 days) white lupine plants (*Lupinus albus*) (Page *et al.*, 2006). Nevertheless, Mn was present in a large amount in the root system, hypocotyls and stem in older lupine plants, immediately after the labeling phase (day 0). Seven days later (day 7) almost all ^{54}Mn had moved to the youngest fully expanded leaves and only a small fraction to the other leaves. Mn accumulation was observed in the periphery of the oldest leaves. These authors reported that Mn was rapidly released from the roots into the xylem,

reaching photosynthetically active leaves via the transpiration stream. Furthermore, the low mobility of this element via phloem in the shoot may be due to a restricted loading of soluble Mn into the phloem or by insolubilization in the leaves, although the issue remains to be clarified (Page *et al.*, 2006). Page and Feller (2005) emphasized that little is known about the mechanisms involved in the loading of Mn into the phloem and the chemical transport forms.

In addition to the long distance transport of Mn, short distance transport mechanisms are important for the translocation of this metal into the cell and cell organelles. These mechanisms involve Mn translocation throughout the plasma membrane and the biomembranes of organelles (Ducic and Polle, 2005; Pittman, 2005). Possible mechanisms of homeostasis and Mn transport, based on studies performed in yeast (*Saccharomyces cerevisiae*) cells and in *Arabidopsis thaliana* plants, have been discussed (Delhaize *et al.*, 2007; Reddi *et al*., 2009). They pointed out that transport proteins play an important role for the maintenance of adequate Mn concentrations in the cytoplasm. Moreover, a variety of metal transporter family proteins with a broad-specificity such as Fe^{2+} and Ca^{2+} transporters have also the ability to transport Mn into the plant cells (Pittman, 2005). Migocka and Klobus (2007) demonstrated the activation of an antiport system in *Cucumis sativus* with different affinities for Pb, Mn, Ni and Cd in the root plasma membrane. This antiport system participates as part of the general defense mechanism activated under heavy metal stress. Using tonoplast-enriched vesicles, Shigaki *et al*., (2003) suggested that a Cd/H antiporter might also be involved in Mn accumulation in vacuoles.

In Table 1 we have summarized some of the metal transporter proteins, their families, cellular localization and the transported metals. Transporter proteins are described according to their localization. In the plasma membrane, four Mn^{2+} uptake transporters are identified: AtIRT1, Nramp, AtYSL (Ducic and Polle, 2005; Pittman, 2005) and PHO84 (Ducic and Polle, 2005). IRT1 can transport Mn when expressed in yeast (Korshunova *et al*., 1999). Nramp is also considered a metal transporter protein that can transport Mn away from other cations. This protein may be localized in the tonoplast rather than in the plasma membranes (Thomine *et al*., 2003). AtYSL belongs to the yellow stripe-like (YSL) proteins, also involved in metal-complex transport in the plasma membrane (Roberts *et al*.,

2004). This complex can be formed by nicotianamine (NA), which is a strong chelator of metals including Mn^{2+} (Pittman, 2005). PHO84 is a transporter protein identified in *S. cerevisiae* and it has a high-affinity phosphate uptake (Mitsukawa *et al*., 1997). Luk *et al*. (2003) reported a new form in Mn transport as $MnHPO_4$. However, there is no evidence for Mn^{2+} or $MnHPO_4$ accumulation by a plant phosphate transporter (Pittman, 2005).

Some transport proteins have been related to Mn^{2+} transport and accumulation into the intracellular compartments, such as the vacuole. It has been suggested that a metal transporter (specifically antiporter CAX2, calcium exchanger 2) originally identified as a Ca^{2+} transporter (which can also transport Cd^{2+}) located in the cytosol. It has also the ability to transport Mn to the vacuole in tobacco plants (*Nicotiana tabacum*) and yeast (Hirschi *et al*., 2000; Pittman, 2005) (Table 1). ATP-binding cassette (ABC) protein transporters are considered to be involved in detoxification processes (Martinoia *et al*., 2002). Studies on cyanobacteria also suggested the putative role of these proteins in Mn^{2+} transport (Bartsevich and Pakrasi, 1996).

Another protein, considered indirectly as a metal transport protein is the ShMTP1, which is able to sequester metal ions within cells or efflux them out of the cells (Delhaize *et al*., 2003). Therefore, these authors considered it a metal-tolerant protein. Ducic and Polle, (2005) and Pittman (2005) highlighted that, despite the available information about Mn transport across membranes in plant cells, the Mn transport and efflux strategies into the mitochondria, chloroplasts and Golgi are not completely understood. Nonetheless, Mills *et al*. (2008) have recently identified a Ca-ATPase that also transports Mn^{2+} into Golgi apparatus (Table 1).

2.4 Manganese as an essential element in plant metabolism

The main Mn role in photosynthesis is its involvement in the water-splitting system of photosystem II (PSII), which provides electrons necessary for photosynthetic electron transport. In water photolysis, a group of four Mn atoms (Mn cluster) is associated with the oxygen evolving complex (OEC) bound to the reaction center protein (D1) of PSII (Goussias *et al*., 2002). The Mn cluster in PSII accumulates four positive charges, which

oxidize two water molecules, releasing one O_2 molecule and four protons. Therefore, this metal cluster is considered a catalyst compound of water oxidation (Zouni *et al*., 2001), where Mn ions are close to a redox-active tyrosine residue (Z and D) (Goussias *et al*., 2002).

Manganese also plays a role in ATP synthesis (Pfeffer *et al*., 1986), in RuBP carboxylase reactions (Houtz *et al*., 1988) and the biosynthesis of fatty acids, acyl lipids and proteins (Ness and Woolhouse, 1980). In addition, Mn plays a primary role in the activation and as cofactor of various enzymes in plants (~35) (Burnell, 1988), such as: Mn-superoxide dismutase, Mn-catalase, pyruvate carboxylase and phospho-enolpyruvate carboxykinase (Ducic and Polle, 2005). Manganese is also essential for the biosynthesis of chlorophyll (through the activation of specific enzymes), aromatic amino acids (tyrosine), secondary products, like lignin and flavonoids (Lidon *et al*., 2004). It also participates in the biosynthetic pathway of isoprenoids (Lidon *et al*., 2004) and assimilation of nitrate (Ducic and Polle, 2005). Hence, Mn is involved in metabolic processes such as respiration, photosynthesis, synthesis of aminoacids and hormone activation (indol acetic acid, IAA) throughout the IAA-oxidases (Burnell, 1988).

Table 1. Some transporter proteins implicated in Mn^{2+} and other cations transport and their cellular localization. (Summarized from Ducic and Polle, 2005; Pittman, 2005 and Mills *et al*., 2008). Abbreviations as follows: At= *Arabidopsis thaliana*; Sh= *Stylosanthes hamata*).

Transporter proteins	**Protein cellular localization and protein family transporters**	**Transported ions**
AtIRT1	Plasma membrane protein (ZIP, zinc-regulated transporter/iron regulated transported (ZRT/IRT1) related protein) family transporter	Mn^{2+} and Fe^{2+}, Zn^{2+} and Cd^{2+} under Fe-deficiency conditions.
AtECA1	Endoplasmic reticulum (ER) Ca^{2+}- and Mn^{2+}-transporting P-type ATPase	Ca^{2+} and Mn^{2+}
AtCAX2	Vacuolar cation/H^{+} antiporter CAX (the cation exchanger)	Mn^{2+}, Ca^{2+} and Cd^{2+}
AtNramp3	Vacuolar Nramp transporter. Also, possible plasma membrane localization (Nramp?)	Mn^{2+}, Fe^{2+} and Cd^{2+} in Fe-deficiency conditions
ShMTP1	Vacuolar-localized cation diffusion facilitator (CDF) family transporter	Related to the *Stylosanthes hamata* Mn^{2+} transporter
ABC	Vacuolar- localized ATP binding cassette transporter families	Related to the cyanobacterium *Synechocystis* Mn^{2+} transporter
AtOPT3	Probably located in the plasma membrane (AtOPT3?). It is an oligopeptide transporter-like protein (OPT).	Possible transport of Cu^{2+} and Fe^{2+} and Mn^{2+}
AtYSL	Probably located in the plasma membrane. Yellow stripe-like transporter with equivalent function to rice OsYSL2, a Mn^{2+}-nicotianamine (NA) and Fe^{2+}-NA transporter	Mn^{2+} and Fe^{2+}
AtECA3	Ca^{2+} transporters (Ca-ATPases) in Golgi.	Mn^{2+} and Ca^{2+}
PHO84	Probably located in the plasma membrane. It is $MnHPO_4$ transporter.	Mn binding to phosphate.

As a cofactor of superoxide dismutase (SOD), manganese participates in the plant's defense against oxidative stress, produced by elevated levels of activated forms of oxygen and free radicals (reactive oxygen species, ROS), which are harmful to plants. It has been proposed that Mn can act as a scavenger of superoxide ($O_2^{\cdot -}$) and hydrogen peroxide (H_2O_2). However, this mechanism is still unclear (Ducic and Polle, 2005).

Manganese SOD (MnSOD) belongs to the group of metal-containing SOD enzymes which are classified according to their metal cofactor: iron SOD (Fe-SOD), localized in the chloroplast; copper-zinc SOD (Cu/Zn-SOD), located in the chloroplast, cytosol, and possibly in the extracellular space. Manganese SOD is found mainly in mitochondria (Clemens *et al*., 2002) and peroxisomes (Alscher *et al*., 2002). MnSOD may play an important role in the adaptive responses of plant cells under environmental stresses such as salt stress, enhancing their tolerance. This tolerance has been demonstrated in transgenic plants of *Arabidopsis*, where Mn-SOD was overexpressed (Wang *et al*., 2004). Similarly, in tomato transgenic plants which overexpressed MnSOD, an improvement in tolerance to NaCl stress in seedlings was concomitant with an improvement in seed germination and root development (Wang *et al*., 2007).

2.5 Manganese phytotoxicity and injury symptoms

As an essential micronutrient, low Mn levels are absolutely necessary for normal nutrition and development of plants. Normal Mn contents of leaves differ greatly between species (30-500 mg kg^{-1} Mn dry mass, Clarkson, 1988). Nonetheless, when it is present in excessive amounts, it is extremely toxic to plant cells (Migocka and Klobus, 2007). The injury extent of Mn toxicity is approximately proportionate to the concentration of accumulated Mn excess. However, there is considerable inter- and intra- specific variation among Mn levels that induce toxicity as well as the symptoms of this toxicity in plant species (Foy *et al*., 1988).

In addition to a decrease in growth rate, symptoms of Mn toxicity such as chlorosis in leaves (intervenial and marginal) and necrotic leaf spots are very common and have been reported in the whole plants of canola (Moroni *et al*., 2003), clover (Rosas *et al*., 2007),

ryegrass (Mora *et al*., 2009) as well as in leaves of barley and cowpea (Demirevska-Kepova *et al*., 2004; Führs *et al*., 2008) (Table 2). Necrotic brown spots and chlorotic leaves are frequently reliable indicators of the severity of Mn toxicity in plants (Wissemeier and Horst, 1991). The intervenial chlorosis due to Mn toxicity can have an appearance similar to that observed under Fe deficiency (Sarkar *et al*., 2004). Moreover, the Mn toxicity is intensified when other available elements such as Ca, Mg, K, Fe and Si are in a low quantity (Abou *et al*., 2002). However, a decrease in productivity by Mn toxicity without the appearance of leaf visual symptoms is sometimes observed (Miner and Sims, 1983). It is important to know that all these symptoms induced by Mn toxicity are preceded by an alteration of the photosynthetic apparatus and the photosynthetic performance of plants.

Other studies have shown that in rice (*Oryza sativa* cv. Safari) exposed to Mn excess in a nutrient solution, Mn was predominantly accumulated in leaves compared with roots (Lidon, 2001), whereas in *Sinapis alba* Mn mostly accumulated in the shoots (Farasova and Beinrohr, 1998).

Table 2. Symptoms of Mn toxicity and Mn concentrations in organs of some plant species subjected to toxic Mn concentrations according references from the last decade (earlier references in the text). Mn treatments were performed in nutrient solutions with $MnCl_2$ or $MnSO_4$.

Species	Mn treatment	Mn concentrations in different plant organs	Symptoms of Mn toxicity	References
Rice (*Oryza sativa* L.)	583 µM	Shoots: 2020 µg g^{-1} dw	Decrease of shoot growth rate.	Lidon and Texeira (2000a)
Barley (*Hordeum vulgare* L.)	1830 µM	Leaves: 656 mg g^{-1} dw	Dark-brown necrotic spots, individually or in groups.	Demirevska-Kepova *et al.* (2004)
	18300 µM	Leaves:1615 mg g^{-1} dw		
Ryegrass (*Lolium perenne* L.)	355 µM	Shoot: 2357 mg kg^{-1}	Chlorosis and necrotic leaves.	Rosas *et al.* (2007)
		Roots: 2408 mg kg^{-1}	Decrease of dry weight in roots.	
Ryegrass (*L. perenne* L.)	150 µM	Shoot: 902 mg kg^{-1}	Dry weight reduction.	Mora *et al*. (2009)
		Roots: 1342 mg kg^{-1}	Dry weight reduction.	
Clover (*Trifolium repens* L.)	355 µM	Shoot: 2050 mg kg^{-1}	Reddish borders on leaves.	Rosas *et al*. (2007)
		Root: 7481 mg kg^{-1}	Decrease of dry weight.	
Soybean (*Glycine max* L.)	200 µM	Leaves: 806 mg kg^{-1}	Chlorotic leaves.	Lavres Jr *et al.* (2009)
		Roots: 502 mg kg^{-1}	No visual symptoms, increase in root diameter.	
Cowpea (*Vigna unguiculata* L.)	50 µM	Leaves: ~ 25 µmol g^{-1} dw	Brown spots on leaves.	Führs *et al.* (2008, 2009)
Canola (*Brassica napus* L.)	200 µM	Shoot: ~ 3500 µg g^{-1} dw	Necrotic leaf spots, chlorosis in leaf margin.	Moroni *et al*. (2003)
Juncus effuses L. (wetland plant)	500 µM	176 mg kg^{-1}	Reduction in plant dry biomass and height, no phytotoxic visual symptoms.	Najeeb *et al*. (2009)
Populus cathayana	1000 µM	Leaves: 713 mg kg^{-1} dw	Decrease in shoot height, total biomass, and total leaf area.	Lei *et al.* (2007)

Despite the importance of Mn excess in the photosynthetic performance of plants, only a few studies about this issue are available. A reduction in photosynthesis, in chlorophyll *a* and *b* contents and their biosynthesis, as well as a reduction in carotenoids is frequently found in plants and also in algae under Mn excess (Macfie and Taylor, 1992; Hauck *et al*., 2003). In rice cultivated at different Mn concentrations (from 2.3 to 583 µM), a significant decrease in chlorophyll *a* content has been reported at the highest Mn concentration (Lidon and Teixeira, 2000a). Nable *et al*. (1988) reported an early inhibition of photosynthesis concomitant with a high Mn accumulation in the leaves in *Nicotiana tabacum* cultivated in nutrient solutions with a Mn excess (1,000 µM). The authors concluded that the inhibition of photosynthesis is an early indicator for Mn toxicity in tobacco leaves. Lidon *et al*., (2004) also observed a decline in net photosynthesis (µmol CO_2 $m^{-2}s^{-1}$) and photosynthetic capacity (µmol O_2 $m^{-2}s^{-1}$) in rice plants subjected to 9.1 and 36.4 µM Mn treatment, respectively, with no changes in the levels of the ratio between variable (Fv) and maximum (Fm) chlorophyll fluorescence (Fv/Fm). However, an increase in the photochemical quenching and the quantum yield of non-cyclic electron transport was found up to 36.4 µM Mn. Since Mn is accumulated in thylakoids, this element may interfere with thylakoid stacking, decreasing net photosynthesis (Lidon and Teixeira, 2000a).

Similarly to Mn toxicity, Mn deficiency also depressed leaf photosynthetic capacity in plants of *Carya illinoinensis*, primarily by reducing the number of PSII units per unit leaf area, but these PSII units maintained abilities similar to those of the control plants (Henriques, 2003). Increased Mn concentrations (0 – 1,000 and 10,000 µM Mn) in wheat plants inhibited the biosynthesis of chlorophyll and carotenoids, inducing a decrease in photosynthetic electron transport rates and therefore a decrease in the rate of photosynthesis (Macfie and Taylor, 1992). Higher Mn concentrations are also involved in a shortening of root and shoot and in a decreased chlorophyll concentration in the Mn accumulator plant, *Alyssum murale* (Abou *et al.*, 2002). Recently, Amao and Ohashi (2008) suggested that high Mn amounts in spinach leaves inhibited the activity of oxygen evolved complex of PSII.

2.5.1 Manganese toxicity and oxidative stress

Mn toxicity can also trigger oxidative stress in plant cells (Demirevska-Kepova *et al*., 2004). As a toxic metal, Mn can cause metabolic alterations and macromolecular damage that disrupt the cell homeostasis (Hegedüs *et al*., 2001; Polle, 2001). According to Lynch and St. Clair (2004), Mn toxicity in plants generates reactive oxygen species (ROS), mainly $OH^{\cdot}$ the most reactive oxidant and harmful species in cells (Lidon and Henriques, 1993). With respect to the oxidative stress responses to Mn excess, Lidon and Teixeira (2000b) reported that at the first growth stages of rice, two kinetic phases can be distinguished: in the first one, there is an increase in Mn accumulation in the thylakoid lamellae (McCain and Markley, 1989), which inhibits electron leakage from the Hill and Mehler reactions, limiting ROS formation. In the second kinetic phase, higher Mn amounts inhibit the non-cyclic photophosphorylation process, promoting an increase in ROS production that parallels an injury increase in the thylakoid peroxidase system (Lidon and Teixeira, 2000b). These authors concluded that Mn excess increases the disorganization in chloroplast lamellae, but elevated activity of superoxide dismutase (SOD) still limits cell damage.

It has also been reported that in *Cucumis sativus* plants both Mn excess and optimum light intensity determine an enhancement in oxidative stress by increased Mn content in the tissues concomitant with an inhibition of plant growth (Shi *et al*., 2006). Investigations performed by González *et al*., (1998) showed that lipid peroxidation was not induced by Mn-toxicity stress in the mature leaves of *Phaseolus vulgaris*, although other studies have shown that lipid peroxidation occurred in isolated chloroplast of wheat (Panda *et al*., 1986). However, González *et al*. (1998) mentioned that this damage process could be related to the development stage of leaves, with the damage being more intense in immature than in mature leaves.

The geographical origin of the species and climatic conditions also affect the degree of Mn toxicity in plant species or populations as shown in two populations of *Populus cathayana*, coming from a wet and dry climate cultivated in an acid solution with increasing Mn concentrations (Lei *et al*., 2007). The results showed that the wet climate population accumulated more Mn in plant tissues especially in leaves, decreasing their growth, chlorophyll contents, and activities of antioxidant enzymes than the dry climate population.

2.6 Tolerance mechanisms to manganese toxicity

The ability of plant for to grow and survive in a metal-contaminated environment, commonly called resistance, can be achieved through different mechanisms: avoidance and/or tolerance. The former involves a protective role that prevents the metal ions from entering the cytoplasm of plant cells (Blamey *et al*., 1986; Marschner, 1991). The latter strategy (tolerance) implies a detoxification of metal ions after they have crossed the plasma cell membrane or internal organelle biomembranes (Macfie *et al*., 1994). However, as shown in the following paragraphs, the differentiation of these mechanisms in the pertinent literature is very confusing.

The sequestering of Mn in the apoplast is considered an avoidance mechanism. However, some researchers have included this feature as a tolerance mechanism. For example, Horst *et al*., (1999) suggested that tolerance to Mn excess in *Vigna unguiculata* is performed by the reduction of Mn^{2+} activity in the apoplast throughout complexation by organic acids. In this species, symptoms such as brown leaf cell spots are also identified as oxidized Mn, and phenolic compounds present in the cell walls are considered a Mn tolerance mechanism (Wissemeier and Horst, 1992). On the other hand, the Mn^{2+} oxidation by peroxidases in the cell walls of roots is considered by Marschner (1991) as an avoidance mechanism, although the existence of such a mechanism was not considered in the study of Horiguchi (1987). In this study, it is suggested that oxidized Mn deposition in plant tissues corresponds to a tolerance mechanism to Mn toxicity, with *Cucumis sativus* being more tolerant to high Mn deposition in tissues than melon (*Cucumis melo)*. Blamey *et al*., (1986) reported the accumulation and secretion of Mn^{2+} in and around the trichomes of sunflower plants (*Helianthus annuus*) as a Mn tolerance mechanism. Another strategy that plants use to prevent the toxic effects of heavy metals as well as of Mn can be the efflux from the cell. In this process, the Mn cell is delivered into the Golgi apparatus and finally exported from the cell via secretory pathway vesicles that carry the metal to the cell surface (Ducic and Polle, 2005).

Summarizing the available literature regarding tolerance mechanisms to Mn, it appears that the main Mn tolerance mechanism is the sequestration by organic compounds in metabolically less active cells or organelles. The vacuole is considered the biggest and most important compartment, because it can store many toxic compounds (Pittman, 2005). Hence, an increase in phenolic compounds was found in the hydrophyte (*Trapa natans*) leaves exposed to high Mn

levels (130 µM) (Baldisserotto *et al*., 2004). These compounds chelate Mn inside the vacuole, segregating the metal ion in the protoplasm and thus reducing the damage (Davis *et al*., 2001). A similar key role has recently been assigned to oxalic acid in Mn internal sequestration by chelating specifically the Mn excess in vacuoles of Mn hyperaccumulator plants (*Phytolacca americana*) (Dou *et al*., 2008). Furthermore, it has been observed that a Mn excess can accumulate dark material in the vacuoles, probably for deposition of Mn oxides or an increase in polyphenol oxidase activity in *Citrus volkameriana* plants (Papadakis *et al*., 2007). Similarly, studies about the effect of Mn excess in varieties of the conifer *Pseudotsuga menziesii* showed dark deposits of Mn-complexes in plant root vacuoles, which were associated with phosphate, establishing "free" Mn^{2+} to form insoluble complexes, giving a greater tolerance (Ducic and Polle, 2007). Additionally, these studies showed that both, root elongation and biomass production were inhibited by Mn treatment above 2,500 µM, mainly in *P. menziesii* var. *glauca*, confirming that it is a species with lower tolerance than *P. menziesii* var. *viridis.*

Another strategy to confer Mn tolerance inside plant cells is associated with several metal transporter proteins identified in the Mn transport mechanisms described in section 4. Metal transporter proteins located in the tonoplast (CAX2 in tobacco plants, ShMTP1 in *Arabidopsis* plants) conferred greater tolerance on elevated Mn^{2+} levels due to the internal sequestering of this element (Hirschi *et al*., 2000; Delhaize *et al*., 2003). Other transporter proteins (ECA1) can maintain low cytosolic Mn, since it moves into the endoplasmic reticulum (Wu *et al*., 2002). In this research, ECA1 was able to reduce cytosolic Mn^{2+}, preventing an interference with the internal distribution of other ions (Mg^{2+}, Fe^{2+} or Ca^{2+}).

Unlike other metal stresses, the accumulation of Mn excess does not have a single cell target. Depending on the plant species, different organelles can serve as stores for this accumulation (Lidon *et al*., 2004). Under high Mn levels apart from vacuoles, chloroplasts are important sinks of this metal in *Citrus volkmeriana*. This feature, together with the larger size of this organelle, is considered to be an adaptive response of this plant to Mn excess (Papadakis *et al*., 2007). In general, cultivated plants like rice are considered tolerant to Mn toxicity (Lidon, 2001) because their leaf tissues can accumulate from 5 to 10 times more Mn than other grasses (Foy *et al*., 1978). The Mn tolerance mechanism in this species included the inhibition of apoplastic influx

from the cortex toward the stele and symplastic Mn assimilation in the shoot protoplast, where the chloroplast is the main target (Lidon, 2001).

The distribution of Mn excess in both roots and shoots is dependent on plant species and genotype. Early research associated Mn tolerance in some plants with a greater retention of Mn excess in the roots, as mentioned by Andrew and Hegarty (1969) in regard to tropical and temperate legume species. The root retention of heavy metals has been attributed to the formation of metal complexes in roots (Foy *et al*., 1978). Metals with high electro-negativity accumulate in roots in larger amounts than metals with low electro-negativity. In the latter instance, Mn and Zn metals can easily be translocated to the tops (Chino, 1981).

In proteomics studies, a comparison of Mn-sensitive and Mn-tolerant cultivars of cowpea (*Vigna unguiculata*.) has shown relevant features of leaf apoplast in the expression of Mn toxicity: formation of brown spots, induction of callose formation and an enhanced release of phenols and peroxidases into the apoplast (Fecht-Christoffers *et al*., 2003; Fecht-Christoffers *et al*., 2006). Specific proteins involved in the regulation processes, such as CO_2 fixation, stabilization of the Mn cluster of the photosystem II, pathogenesis-response reactions and protein degradation, were affected at low or high Mn levels, mainly in the Mn-sensitive cowpea cultivar (Führs *et al*., 2008). Chloroplastic proteins, which are important for CO_2 fixation and photosynthesis, were of lower abundance upon Mn-induced stress, suggesting the scavenging of metabolic energy for a specific stress response. Führs *et al*., (2008) concluded that a coordinated interplay of apoplastic and symplastic reactions seems to be important during the Mn-stress response in plants.

To alleviate metal toxicity in plants, the antioxidant systems are also considered an important tolerance mechanism. The antioxidant systems include antioxidant enzyme "scavengers" such as superoxide dismutase (SOD), catalase (CAT), peroxidases (phenol peroxidase, POX, ascorbate peroxidase, APX, guaiacol peroxidase, GPX) and the non-enzymatic antioxidant molecules: ascorbate, α-tocopherol, carotenoids, flavonoids and glutathione (Foyer and Noctor, 2003; Apel and Hirt, 2004). These constitute other mechanisms against ROS, produced by Mn toxicity.

Higher activities of antioxidant enzymes are found in response to a Mn excess in woody plants (Lei *et al*., 2007), in herbs such as white clover (*Trifolium repens*.) and in ryegrass (*Lolium perenne*), which suggest a lower oxidative stress (Rosas *et al*., 2007; Mora *et al*., 2009). Another

plant tolerance mechanism to metal toxicity is associated with lower metal uptake and translocation to other organs (Hall, 2002). The exudation of organic acid anions (carboxylates) to the rhizosphere may minimize the absorption by roots of such metals as aluminium and nickel (Ma *et al*., 2001; Yang *et al*., 1997). There are few reports regarding the release of organic acid anions by roots in the event of Mn toxicity (González and Lynch, 1999). Recently, Mora *et al*. (2009) reported that the root exudates of oxalate and citrate may decrease Mn availability in the rhizosphere, enhancing their Mn tolerance in ryegrass subjected to Mn toxicity.

Other nutrient applications can help minimize the effects of Mn toxicity. Thus, it has been reported that silica (Si) addition significantly decreases lipid peroxidation caused by an Mn excess decreasing the symptoms of Mn phytotoxicity and improving plant growth in some plants (Iwasaki *et al*., 2002; Shi *et al*., 2005). There is also an association between Mn toxicity and the decrease in Ca concentration in barley plants (*Hordeum vulgare*), indicating a competition and a specific interaction during the absorption and/or translocation of these elements (Alam *et al*., 2001). Another study, in which Ca is applied to reduce the Mn toxicity, showed that Ca additions inhibited Mn translocation from roots to shoots in barley plants, but did not affect the Mn absorption in roots (Alam *et al*., 2006). This suggested that Ca could avoid Mn accumulation in shoots, protecting the photosynthetic apparatus from the dangerous effect of an excess of Mn. In barley, Mn toxicity could also be repressed by high K contents, which inhibit both the absorption and the translocation of Mn (Alam *et al*., 2005).

2.7 Conclusions and perspectives

Manganese is considered an essential micronutrient for the metabolic process in plants. Nevertheless, both deficiency and excess alter these processes. Acid soils make excessive Mn amounts toxic for the plants. Mn toxicity is a world-wide problem in areas with acid soils. This toxicity alters physiological, biochemical and molecular processes at the cell level. It is crucial to know the limitations of these soils for the purpose of soil-plant interaction management, especially in relation to the presence and Mn excess. Thus, the knowledge of Mn uptake, translocation, accumulation and resistance mechanisms in crop plants under Mn excess and toxicity is of great importance to crop improvement.

Most molecular and physiological approaches to Mn transport inside plant cells have recently been analyzed, as these are useful tools for understanding resistance mechanisms. However, the question as to which is the best candidate gene related to Mn toxicity in acid soil continues to elude plant scientists due to Mn toxicity in plants being a complex trait and involving multiple physiological and biochemical mechanisms and a wide array of genes. The understanding of these mechanisms will contribute to improving the yield and quality of cultivated plants in acid soils. Future efforts for developing Mn-tolerant plants should take all these aspects into account.

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Chapter 3:

Effect of Mn excess on biochemical and physiological properties of Highbush blueberry (*Vaccinium corymbosum* L.) cultivars growing in nutrient solution.

Paper in preparation

Effect of Mn excess on biochemical and physiological properties of Highbush blueberry (*Vaccinium corymbosum* L.) cultivars growing in nutrient solution.

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Abstract

Manganese (Mn) is an essential micronutrient for plants. However, when it is in excess, it can lead to negative effects on physiological and biochemical characteristics in plants. Besides, plant species had different resistant mechanisms against abiotic stress such as excess of metals. One year old plants of blueberry cultivars were grown in hydroponic solution with 2, 10, 50, 250, 500, and 1000 µM of Mn treatments for 25 days. We compared the manganese (Mn) excess in three highbush blueberry (*Vaccinium corymbosum* L.) cultivars: Legacy, Brigitta and Bluegold. Parameters evaluated were: plant growth as shoot and root biomass, Mn content, photosynthetic performance (photochemical efficiency of PSII, CO_2 assimilation and stomatal conductance). Lipid peroxidation, radical scavenging activity, superoxide dismutase activity and organic acid anions exudations, were also anlysed. Results showed detrimental effects on plant growth, photochemical efficiency and CO_2 assimilation, as well as in biochemical features. Legacy showed a major Mn content in roots compared with other cultivars, evidencingno changes in photosynthetisis and in antioxidant capacity. Bluegold presented lower levels in net photosynthesis and photochemical processes and an increase of lipid peroxidation. In organic acid anions exudation, oxalate and citrate had greater level of exudation in Legacy and Brigitta, which suggest a major Mn-tolerance in front to an excess of this trace element compared with Bluegold. In conclusion, Legacy cultivar showed a higher Mn-tolerance than Brigitta and Bluegold, being this last cultivar the most sensitive.

3.1 Introduction

Manganese (Mn) is an essential micronutrient for most microorganisms and plants, being indispensable for many physiological and biochemical processes such as growth, photosynthesis, photosynthetic proteins and enzymes among others (Marschner, 1995). Depending on its soil content and the factors controlling its availability as low pH (< 5) and redox conditions Mn can be toxic for plants (Rengel 2000; Millaleo et al 2010). A decreasing pH leads to an increment of available toxic manganese (Mn^{2+}) in the soil solution which can be readily transported into the root cells and translocated to the shoots, where it is accumulated (Marschner, 1995).

Manganese toxicity is one of the most important factors limiting plant growth, especially in tropics and subtropics areas (Horst, 1988). Many studies showed that under Mn excess physiological and biochemical plant functions, like growth (Lei et al., 2007), and photosynthetical performance including photosynthetic pigments are impaired (Hauck et al., 2002, 2003, 2006; Macfie y Taylor, 1992). In addition, under Mn toxicity reactive oxygen species (ROS) can be exacerbated conducting to oxidative stress and lipid peroxidation membranes as reported in barley, clover, and perennial ryegrass (Demirevska-Kepova et al., 2004; Rosas et al. 2007, Mora et al. 2009, respectively). To overcome with Mn induced oxidative stress plants have developed some mechanisms like an increase of antioxidant enzymes activity [superoxide dismutase (SOD) and peroxidase (POD)] as reported in cucumber (Shi et al., 2006), perennial ryegrass (Mora et al., 2009) and pea (Gangwar et al., 2010) exposed to a Mn excess. The exudation of organic acid anions (carboxylates) by apical roots is another important mechanism to counteract the deleterious effects of toxic cations. Carboxilates are able to reduce the concentration of toxic cations in the rizosphere or accumulating them in the cytoplasm of plant cells (Ryan et al., 2001). In ryegrass and white clover (Rosas et al., 2007) showed that toxic Mn is excluded from the roots by the exudates (exclusion mechanism) and kept out of it, forming soluble or insoluble complexes that decrease Mn availability (Wang and Mulligan, 2009). In ryegrass seedlings, Mora et al., (2009) found that oxalate and citrate were the most abundant carboxylates exudates induced by Mn excess, mainly in the most Mn resistant cultivars.

In southern Chile, acid soils of volcanic origin are common. In these soils a serious constraint for plant growth are toxic (Al^{3+}) and toxic (Mn^{2+}). In this region highbush blueberry (*Vaccinium corymbosum)* is one of the main cultivated crops. With exception of the work of Rojas-Lillo et al

(2014), who studied some physiological and biochemical responses to toxic Mn and UV-B on this crop, the root exudation of carboxylic acids under Mn excess and their association with antioxidant mechanisms are not available. Therefore, the aim of this work is to clarify the implication of the exudation of carboxylic acids and antioxidant responses under Mn excess in *V. corymbosum.*

3.2 Materials and Methods

Plant material and growth conditions.

Three cultivars of highbush blueberry (*Vaccinium corymbosum* L.): 'Legacy', 'Brigitta' and 'Bluegold', were used in this study. One-year old plants of these genotypes coming from a nursery of Temuco, Chile, growing in a substrate of sawdust:soil:pine needles at a 1:1:1 proportion were established under greenhouse in nutrient solution. For conditioning, seedlings were placed in plastic boxes with 10 L of Hoagland's nutrient solution (Hoagland and Arnon, 1959) for 2 weeks. Later, blueberry seedlings were placed in a new Hoagland's nutrient solution in plastic boxes (4 plants per box) and Mn treatments were applied. The time of exposition to Mn excess was 25 days. Blueberry plants were subjected to the following Mn concentrations (added as $MnCl_2$ x $4H_2O$): 2 µM (Control), 10, 50, 250, 500 and 1000 µM Mn. The solutions were daily adjusted to pH 4.8±0.02, where volume was restored to its original level, and were renewed every 1 week. The Hoagland solution used included the following concentrations: 2 mM $Ca(NO_3)_2$, 3 mM KNO_3, 1 mM $MgSO_4$, 0,1 mM KH_2PO_4 , 22 µM H_3BO_3, 2 µM $MnSO_4$, 1 mM NH_4NO_3, 0,07 µM $(NH_4)_6Mo_7O_{24}$, 1,6 µM $ZnSO_4$, 0,4 µM $CuSO_4$, 20 µM Fe- EDTA. Experimental conditions used were under semi-controlled environmental conditions in a naturally lit greenhouse, with a temperature range of 25/20 ºC (day/night), relative humidity range of 50-80%, the photoperiod of 16/8 h (day/night), an average PPF (photosynthetic photon flux) of 300 µmol m^{-2} s^{-1}.

Dry weight and Mn content measurements

At the beginning of Mn treatments, the fresh weight of whole plants from each cultivar was determined. From these plants, a group was separated for dry weight determinations. At the end of the experiment (25 d), plants of blueberry cultivars were harvested and separated in roots and shoots, for fresh and dry weight determinations. Shoots and roots samples were dried in a forced-

air oven for 48 h at 65°C for dry weight determinations. To determine Mn concentrations, first leaves from the first to the fourth node of shoots and roots were dried at 65°C in a forced-air oven for 48 h. Later, samples were ashed in a muffle furnace at 500°C for 8 h and treated with 2M hydrochloric acid (HCl). Mn was quantified using a simultaneous multielement atomic absorption spectrophotometer (Model 969 atomic absorption spectrometer; Unicam, Cambridge, UK) according to Sadzawka et al. (2004) methodology. To obtain the Mn contents, the Mn concentration was multiplied with the respective dry weight for leaves and roots.

Photosynthetic performance

It was analyzed by measuring the photochemical efficiency of the PSII and net photosynthesis (*Pn*). For the photochemical efficiency of PSII, the leaf chlorophyll fluorescence from the second to fourth node of shoots (previously dark adapted for 20 min) was measured using a portable pulse-amplitude modulated fluorimeter (FMS 2; Hansatech Instruments, King's Lynn, UK) according to (Reyes-Díaz et al. 2009). The maximal photochemical efficiency of PSII (Fv/Fm), effective photochemical efficiency of PSII (ΦPSII) as indicator of the effective quantum yield of PSII and non-photochemical quenching (NPQ) were estimated as described by Maxwell and Johnson (2000). The net photosynthesis were performed in attached leaves with similar localization as those used for the determination of fluorescence parameters. The measurement of net CO_2 assimilation was made using a portable CO_2 infrared gas analyzer (Licor LR6400). This analyzer was equipped with a fluorescence leaf cuvette, which controlled the light source (300 µmol $m^{-2}s^{-1}$), temperature, humidity and CO_2 external air obtaining a concentration reference of 360 ppm, with a flow rate of 200 mL min^{-1} and 80% external relative humidity. The temperature inside the leaf chamber was maintained at 20ºC. Stomatal conductance (g_s) was also given by the *Pn* determination.

Lipid peroxidation and Radical scavenging activity

The level of lipid peroxidation (LP) was assessed in fresh samples by monitoring the thiobarbituric acid reacting substances (TBARS) as an index of oxidative damage in plant cells. The absorbance was measured at 532, 600 and 440 nm in order to correct the interference generated by TBARS-sugar complexes according to the modified method (Du and Bramlage, 1992). This method determines the occurrence of malondialdehyde (MDA) as secondary

byproduct of the oxidation of polyunsaturated fatty acids that can react with thiobarbituric acid (TBA) (Hodges et al. 1999) The unit for LP was determined as equivalents of malondialdehyde (MDA) contents (nmol g^{-1} FW). The radical scavenging activity (RSA) of roots and leaves was measured at the end of Mn treatments (25d) using the method of free radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging, as described by Chinnici et al. (2004), with some modifications. The absorbance was measured at 515 nm using Trolox as the standard. The unit used was Trolox equivalent per gram of fresh weight (TE [mg g^{-1} FW]).

Superoxide dismutase (SOD) activity. For determination of the superoxide dismutase (SOD, EC 1.15.1.1) enzyme activity, samples of fresh leaves and roots were frozen in liquid nitrogen and stored at -80°C until extraction of SOD. The extraction procedure was performed as described by Mora et al. (2009). SOD activity was assayed by monitoring the photochemical inhibition of nitroblue tetrazolium (NBT) as described by Giannopolitis and Ries (1977) with minor modifications (Mora et al., 2009). Enzymatic activity of SOD values were standardized by the total protein content, according to Bradford (1976). One unit of SOD activity (U g^{-1}) was defined as the amount of enzyme required to cause 50% inhibition of the reduction of NBT as monitored at 560 nm.

Analysis of root exudates.

Root exudates were collected 25 d after the beginning of Mn treatments, according to Rosas et al. (2007). Intact blueberry plants coming from Mn treatments assays were rinsed carefully with deionized water and transferred to pots containing deionized water (250 mL). For collection of exudates from the whole root system, roots were submerged during 2 h into aerated deionized water and then, subsamples of control and Mn-treated plants were collected, stored and frozen at -20 °C. The organic acid anions (oxalate, malate, citrate and succinate) exudated from roots were quantified by chromatographic analysis. Root exudate subsamples were concentrated by lyophilisation, the residue re-dissolved in 600–800 μL of deionized-sterile water for HPLC injection and filtered (0.22 mm). The chromatographic separation was realized in an HPLC system (Merck-Hitachi model L-4200) equipped with a UV–visible detector and a Sphere Column Heater (Phenomenex Terma model TS-130) according to Meier et al (2012). The flow rate was 1 mL min^{-1} and injected samples were detected at wavelength of 210 nm. Identification

of organic acids anions was determined comparing the retention times and by addition of standards for each organic acid anion.

Experimental design and statistical analyses.

The experimental design is a split-plot design with 3 cultivars x 6 Mn treatments x 4 replicates for the physiological and biochemical determinations. Data were tested in their normality (Shapiro-Wilk test) and equal variance by Kolmogorov-Smirnoff test and then data were analyzed by two-way ANOVA. Significant differences among means values were established by using the multiple comparisons test of Tukey's ($P < 0.05$). All analyses were performed with Sigma Stat 2.0 software (SPSS, Chicago), where differences between the values were considered significant at $P \leq 0.05$.

3.3 Results

Shoots and roots dry weight

The lowest values in dry weight of shoots under increasing Mn treatments, were exhibited in Bluegold, where a consistent decrease (~50%) was observed until 50 µM Mn, where values were constant up the highest dose of Mn (1000 µM) in comparison to control ($P < 0.005$) (Fig. 1A). Legacy and Brigitta manifested also a parallel tendency to decrease under Mn treatments. In roots, no significant differences in dry weight of all cultivars subjected to Mn treatments were exhibited (Fig.1B).

Mn contents in leaves and roots. The Mn content in leaves and roots showed a statistically significant interaction between cultivars and Mn treatments ($P < 0.001$). In leaves, Legacy and Brigitta showed a gradual increment, mainly with the highest Mn treatment (68 and 72%, respectively) compared with control. Instead, Bluegold had significant increment in leaves Mn content only with the highest Mn treatment (1000 µM) with 52% respect to control (Fig. 2). Brigitta had the major Mn content with the highest Mn dose compared with other cultivars. In roots, the most noticeable increment in Mn contents was found in Legacy, where a significant increase from 50 to 1000 µM Mn was evidenced, and where the highest root Mn adquisition of this cultivar was obtained at 500 and 1000 µM Mn (83 and 89%, respectively, compared to control). Brigitta had significant increase in Mn contents with the highest Mn treatment compared

with the control (57%), while Bluegold showed significant differences from 250 to 1000 µM Mn treatment, with a 58% and 74% of increase compared with the control, respectively.

Photosynthetic performance

The maximal photochemical efficiency of PSII (Fv/Fm) and the effective photochemical efficiency of PSII (ΦPSII), showed a significant decrease in Brigitta and Bluegold but not in Legacy, specially at the higher Mn doses compared with their controls ($P > 0.005$, Table 1). Regardeless to ΦPSII, Bluegold and Brigitta have lower values than Legacy at higher Mn doses (500 and 1000 µM Mn). Values of NPQ in Legacy were lower than those of Brigitta and Bluegold, and significantly it decreased (53%) at 500 µM Mn compared with control. Bluegold diminished NPQ values significantly from 10 µM Mn to 250 µM Mn and at the highest Mn doses. Moreover, Brigitta exhibited no significant differences in NPQ between Mn treatments. Indeed, Legacy achieved the lowest values of this parameter concomitant with generally higher ΦPSII values.

Exposure of plants to Mn excess brought to a decrease in the net photosynthesis (*Pn*) in Legacy and Bluegold (Fig. 3). Legacy had a significant reduction from the 50 µM Mn to the highest Mn treatment, with percentages of reduction around 56% (50 µM), 41% (250 µM), 48% (500 µM) and 37% (1000 µM), when they were compared with the controls. Bluegold also exhibited a decrease of *Pn* which ended with a notorious fall at the highest Mn treatment (~80% decrease) ($P \leq 0.005$) respect to control. Significant reductions in stomatal conductance (g_s) values were found in Bluegold and Brigitta particularly with the highest Mn dose. Differently, Legacy decreased only in the intermediate Mn treatments (250 to 500 µM Mn) without change respect to the control at the higher Mn dose.

Lipid peroxidation and radical scavenging activity

Analysis of leaves and roots lipid peroxidation (LP), which indicates a damage of cell membranes were performed. In leaves, Brigitta and Bluegold showed an increasing trend of LP by incrementing Mn doses, mainly at the highest Mn dose compared with the control, while Legacy did not show significant changes (Fig. 4). In roots, both Legacy and Brigitta did not have a significant increment of MDA values in all Mn treatments, nevertheless Bluegold showed an increase of MDA values with higher Mn doses.

In addition, antioxidant activity, measured as radical scavenging activity (RSA) in leaves of Legacy and Bluegold exhibited an increase with increasing Mn treatments. However, measurements on Bluegold had significant high values from 50 to 1000 µM Mn compared with the control (35 to 46%). Instead, Legacy only had a significant increment at the highest Mn dose (1000 µM) respect to control (28%, Fig. 5). It is noteworthy that in roots, cultivars had lower values of RSA than leaves.

Regarding to the antioxidant capacity, the SOD activity in leaves of Legacy remained stable whereas Brigitta had statistically significant decreases in the SOD activity from 50 µM to the latest Mn supplies tested (Fig. 6). In roots, only Bluegold showed a significant gradual reduction in SOD activity, mainly at the 1000 µM Mn compared with the control.

Root exudation of carboxylates

Roots showed a significant increase of oxalate and citrate exudates under Mn supply mostly in Legacy and Brigitta (Fig. 7). It should be noted that, oxalate was the highest organic acid anion exudate, with values around 0.5 to 2 µmol g^{-1} h^{-1}. Besides, Legacy exhibited a significant increment of this acid anion from 500 µM (85% up compared with the control). Bluegold showed no significant increment in oxalate exudation. In contrast, malate exudation in Bluegold and Brigitta generally exhibited a significant decrease at incrementing Mn-treatments.

3.4 Discussion

Mn excess can be an important factor of toxicity in acid soils, affecting the physiological and biochemical features of crop plants (Marschner, 1995), generating different resistance responses even in the same species, genotypes or cultivars (Foy et al. 1995; Fecht-Christoffers et al. 2003). In our research, significant DW reductions were highlighted only in Bluegold shoots, even at the lowest Mn treatment (10 µM; Fig. 1). Reductions of dry weight (DW) due to a Mn excess have been reported in rice (Horiguchi, 1988), ryegrass (Mora et al., 2009), lucerne (Gherardi and Rengel, 2003), pummel (Li et al., 2010), *Cucumis* species (Shi et al., 2005; 2006), maize (Stoyanova et al., 2009) and barley (Alam et al., 2006). Detrimental effects of Mn excess in ryegrass and clover, where DW reductions were higher for shoots than roots were mainly associated with deficient or low P supply (Rosas et al., 2007; Rosas et al., 2011). A major injury in shoots than roots were associated to a Mn toxicity in Mn-sensitive maize cultivar, differing

with other metals behavior (i.e. copper and aluminum), which reduces significantly DW at root level (Doncheva et al., 2009). Additionally, no significant changes were found in DW roots in all blueberry cultivars (Fig. 1), which could support the fact that effects of Mn excess are mainly a reduction in shoots more than roots in this woody plant. With respect to Mn contents, shoots and roots increased significantly and gradually with Mn supplies in all cultivars (Fig. 2). Similar results were obtained by Mora et al. (2009), where a large translocation from roots to shoots was shown by Mn-sensitive ryegrass cultivars, whereas the tolerant ones accumulate more Mn content in roots than shoots. These results suggest an internal mechanism of control on Mn content and lower Mn translocation to shoots in tolerant ryegrass cultivar than in Mn-sensitive cultivar, which had a great translocation to shoot (Mora et al. 2009). Similar tendency was showed in *Citrus grandis* plants subjected to Mn supply where the majority of Mn was retained in the roots with a subsequent low Mn content in aerial tissues implying that a Mn-excess might contribute to a tolerance for Mn toxicity (Li et al., 2010). Recently, Yao et al. (2012a) prove that Mn in woody plants, was readily transferred to above-ground plant parts in chestnut rose, plant considered sensivity to Mn excess, compared to grape plants (Mn tolerant species), which Mn is stored in the root system, which confer a high Mn tolerance. Additionally, these authors suggested that a restriction of xylem loading can be considered as a limiting process for root-to shoot translocation. Considering the above results in other plants, our results showed that Legacy presented a major Mn accumulation tendency in roots, suggesting a Mn tolerance in this cultivar.

Our findings about maximal photochemical efficiency of PSII (Fv/Fm), the effective photochemical efficiency of PSII (ΦPSII) in leaves of blueberry cultivars subjected to Mn excess exhibited a significant decrease in Brigitta and Bluegold with higher Mn dose (Table 1). Researches in rice with cadmium (Cd), suggest photoinhibition by loss the PSII efficiency, due to a decrease of Fv/Fm (Pagliano et al., 2006). Excess Mn also decreased significantly ΦPSII in cucumber (*Cucumis sativus* L.) seedlings, suggesting that lead to a decline of carbon metabolism capacity and/or a low consumption of ATP and NADPH in the Calvin cycle (Feng et al., 2009; Kitao et al., 1997). NPQ values of blueberry cultivars showed a significant decrease in Bluegold from the lower Mn doses treatment, although there was not change in higher Mn doses, remaining almost constant along the time in the others cultivars (Table 1). Doncheva et al. (2009) found a decrease in Fv/Fm and an increase in NPQ values in the Mn-sensitive cultivar of maize, suggesting a photoinhibition by Mn excess. Although high NPQ values reflect heat dissipation in

PSII (Maxwell and Johnson, 2000), sometimes it is not enough to protect efficiently the photosynthetical organelles, as thylakoids and chloroplast (Doncheva et al., 2009). In this sense, net photosynthesis (*Pn*) and stomatal conductance (*Gs*) of Bluegold were negatively affected by excess Mn, mainly with the highest Mn treatment. Legacy had also a significant reduction in *Pn*, but without changes in *Gs* values. On contrary, *Pn* in Brigitta remained stable, but with a significant decline of *Gs* (Fig. 3). Stoyanova et al. (2009) observed a negative relation between Mn leaf concentration and net photosynthesis and stomatal conductance in Mn-sensitive maize cultivars. However, these authors found that in Mn-tolerant maize cultivars, the Mn leaf concentrations increased along with transpiration rate, stomatal conductance and photosynthesis. Thses results suggest that Mn leaf accumulation could be performed by the transpiration stream; leading to that Mn can be transported by xylem from roots. Early experiments in tobacco young leaves grown in nutrient solution with Mn excess showed a decrease in net photosynthesis (Nable et al., 1988), although this decline is not a consequence of decreased leaf CO_2 conductance in leaves. These authors concluded that photosynthesis inhibition is an early indicator of Mn accumulation excess in tobacco leaves (Nable et al., 1988).

It is known that ROS production can modify metabolic pathways and increase protein denaturation, membranes, lipids injury and DNA mutation, even destroy cellular structures (Hegedüs et al., 2001; Najeeb et al., 2009; Desikan et al., 2005). In our results, Legacy did not show changes of lipid peroxidation.In constrast, higher increment of lipid peroxidation had both Brigitta and Bluegold, mainly in leaves with increasing Mn treatments (Fig. 4). It has been demonstrated that a Mn excess can increase lipid peroxidation in cucumber leaves (Shi et al., 2005; Shi and Zu, 2008) and wetland plants (Najeeb et al., 2009), indicating that Mn excess can cause oxidative stress. In addition, Mora et al., (2009) showed that in Mn-tolerant ryegrass cultivars subjected to Mn toxicity, lipid peroxidation was lower than in Mn-sensitive cultivars.On the other hand, these authors also found that Mn-sensitive cultivars incremented shoot Mn concentration concomitant with an augment in lipid peroxidation.

To prevent and reduce damage induced by ROS, plants have developed antioxidant defense mechanisms, which include enzymatic and non-enzymatic systems (Foyer and Noctor, 2003; Apel and Hirt, 2004). Radical scavenging activity (RSA) in blueberry cultivars showed higher differences in leaves than roots with Mn excess. Bluegold increased significantly this activity

starting from 50 µM Mn to the highest Mn-dose in leaves (Fig. 5) and it was proportional to its LP values, reaching maximums at the highest Mn treatment. Interestingly, Legacy had an increase of RSA at the highest Mn treatment, even though lipid peroxidation remained unchanged (Fig. 4). Results with the same blueberry cultivars, but subjected to metal stress (aluminum, Al toxicity) showed that Bluegold is the Al-sensitive cultivar, with the greatest values of lipid peroxidation and lower RSA values (Reyes-Diaz et al., 2010). The authors suggest that Al-tolerant cultivar Legacy can activate defense mechanisms through RSA which contribute to decrease lipid peroxidation values. Likewise, others studies have shown that an enhanced level of antioxidative components can be correlated with increased stress tolerance (Shi et al., 2005). In the same way, it has been suggested that is an increase in superoxide dismutase (SOD) enzyme activity under Mn toxicity, to prevent and mitigate the oxidative damage in plant cells (Srivastava and Dubey, 2011). In our studies, Bluegold had a tendency to decline its SOD activity with the increasing Mn treatment compared with control of leaves and roots. Differently ocurrs in Legacy, where it was unchanged (Fig. 6). Researches carried out with blueberries cultivars subjected to Al toxicity at short time (hours), showed that Brigitta was considered as an Al-resistant cultivar, which reflected an amelioration of SOD activity under these metal stress. This indicates a bigger capacity for controling oxidative stress, compared with Bluegold (Inostroza-Blancheteau et al., 2011). Others studies also confirmed detoxification of Al in barley plants, where SOD activity is involved (Simonovicova et al. 2004).

Organic acid anion exudations in plants are recognized for their chelating capacities of heavy metals decresing the bioavailability of these metals in soils (Najeeb et al. 2009). Exudation of organic acids has different functions, as the uptake of some nutrients as P (Hoffland, 1992; Johnson et al. 1996) and metal detoxification (Jones, 1998). Organic acid anion exudates associated to Mn detoxification are oxalate, citrate, malate and succinate (Horst et al. 1999). Interesting results were observed in roots exudates of organic acids in our experiments (Fig. 7). Of the four organic acid anions analyzed, oxalate presented a higher amount of exudates, compared with others. Similar results were found in ryegrass and white clover grown under Mn excess and acidic conditions, where oxalate and citrate accounted for the most of the carboxylates exudates (Rosas et al., 2007). In our research, Legacy and Brigitta have similar increasing exudation of oxalate, with incrementing Mn treatments. Contrary, in Bluegold there were no

changes in this organic acid exudation. Interstingly, this blueberry cultivar showed the highest values of malate in the control, decreasing drastically with Mn treatments.

Mora et al., (2009) found that Mn-tolerant ryegrass had the highest oxalate exudation rate at increasing excess supply of Mn. Those responses suggest that oxalate can act on internal Mn-tolerance mechanisms. Citrate exudation was found in Mn-tolerant ryegrass, associated to the high affinity of citrate for Mn^{2+} in the rizosphere and high citrate exudation. In our study, Legacy and Brigitta showed an increase in citrate level exudations, compared with Bluegold. Likewise, in researches with wetland plants, Najeeb et al. (2009) showed that citric acid and other organic acids can provided both protons as electrons for reduction of metals in the rizosphere, acting as chelators. Others heavy metals toxicity as lead (Pb) and Cu are associated to Mn tolerance by a release of oxalate in *Arabidopsis* plants and to citrate efflux in rice cultivars (Ryan et al. 2001).

In summary, our results demonstrated that Mn excess affects differently the physiological and biochemical features of blueberry cultivars. Legacy was the most Mn-tolerant cultivar compared to Brigitta and Bluegold, being the last cultivar the most Mn-sensitive. It was due to Legacy cultivar accumulated a high Mn content in roots, maintaining its growth and photosynthesis. Besides, this cultivar showed a higher antioxidant capacity without changes in lipid peroxidation at increasing Mn treatments. Oxalate and citrate were the most important organic acid anions exudated, incrementing its concentration gradually with the enhancement of Mn doses in Legacy.

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Tables and Figures

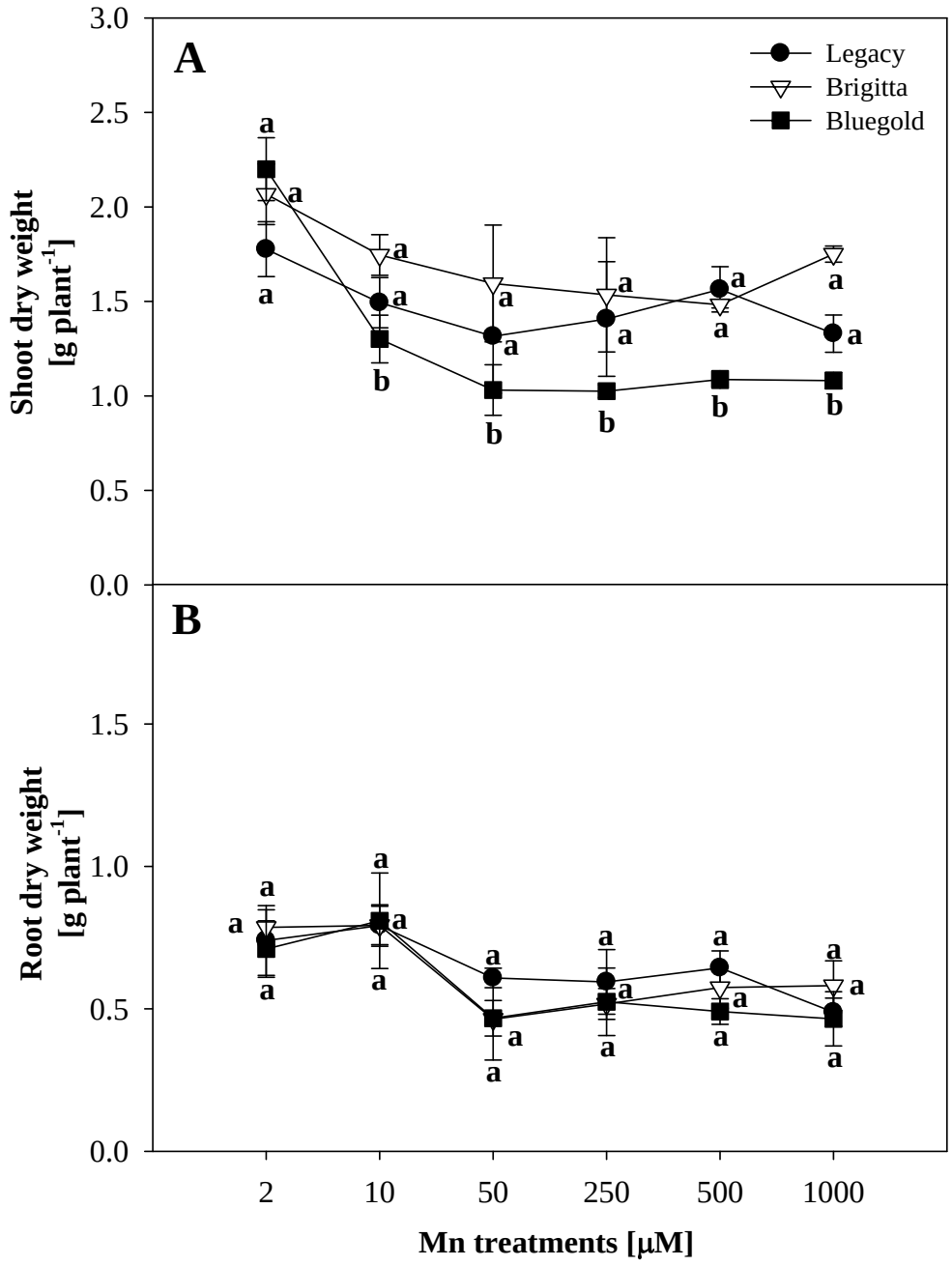

Figure 1. Effect of Mn treatments on dry weight of shoot (leaves + stems) (**A)** and roots dry weight (**B)** in three blueberry cultivars grown in nutrient solution for 25 days. Results are means ± standard error (SE), with n=3. Different lower-case letters indicate significant differences between Mn treatments for the same cultivar ($P \leq 0.05$, Tukey's Test).

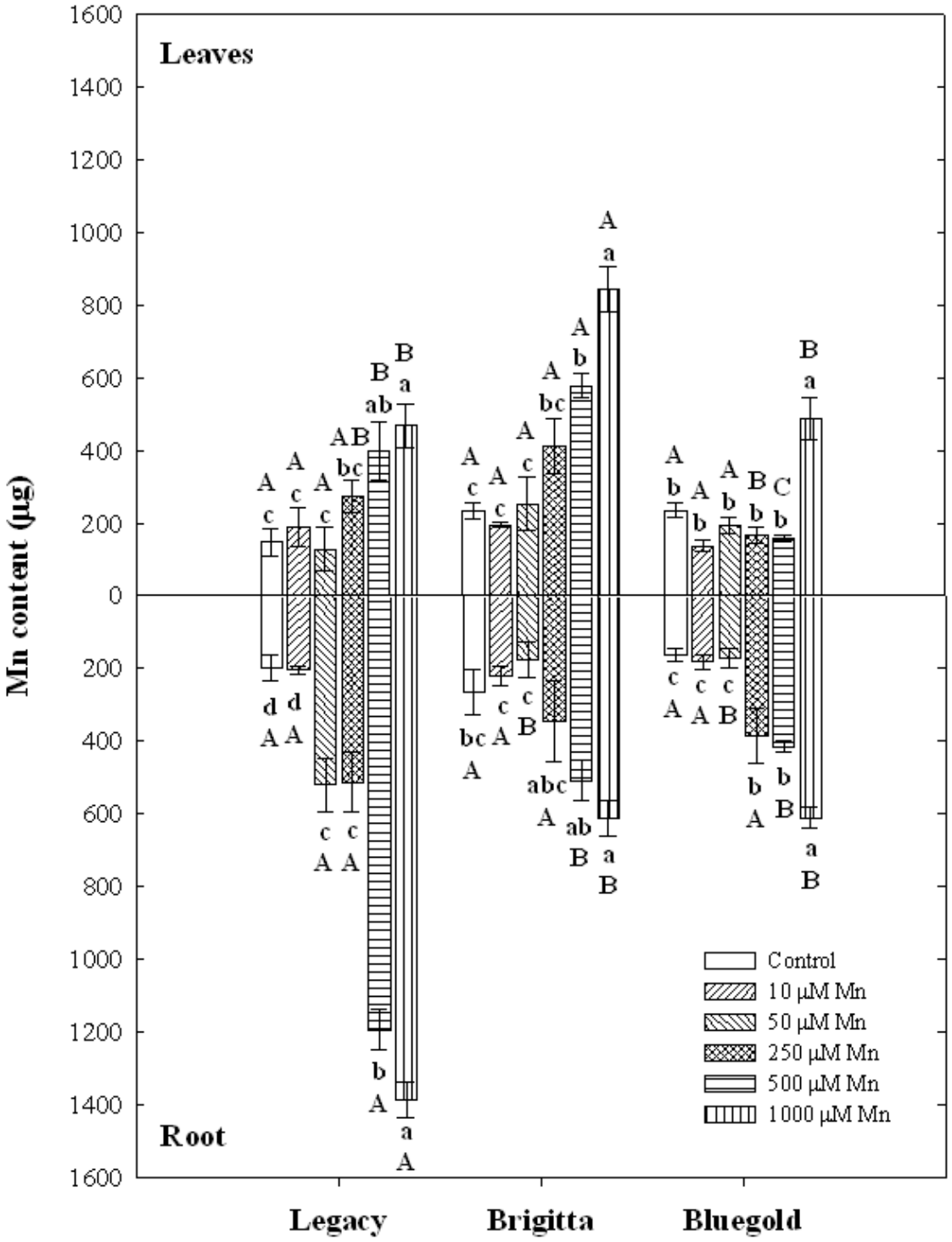

Figure 2. Mn contents in leaves and roots of blueberry cultivars with Mn excess-doses at the end of experiment (25 days). Different lower-case letters indicate significant differences between the Mn treatments for the same cultivar, while different upper-case letters indicate significant differences between the cultivars at a same Mn treatment ($P \leq 0.05$).

Table 1. Maximal photochemical efficiency of PSII (Fv/Fm), effective photochemical efficiency of PSII (ΦPSII) and non-photochemical quenching (NPQ) of three blueberry cultivars subjected to increasing Mn levels (25 days). Different lower-case letters indicate significant differences between Mn treatments at the same cultivar. Different upper-case letters indicate significant differences between cultivars at the same Mn treatment ($P \leq 0.05$).

Cultivar	**Mn treatment [µM]**	**Fv/Fm**	**ΦPSII**	**NPQ**
Legacy	2	0.78 ± 0.005 **aA**	0.115 ± 0.006 **aA**	1.18 ± 0.2 **aB**
	10	0.77 ± 0.008 **aA**	0.103 ± 0.015 **aAB**	1.07 ± 0.2 **aB**
	50	0.78 ± 0.004 **aA**	0.102 ± 0.006 **aA**	1.11 ± 0.2 **aB**
	250	0.78 ± 0.002 **aAB**	0.092 ± 0.006 **aA**	1.04 ± 0.1 **aB**
	500	0.77 ± 0.009 **aA**	0.093 ± 0.013 **aA**	0.55 ± 0.1 **bB**
	1000	0.76 ± 0.008 **aA**	0.089 ± 0.008 **aA**	1.53 ± 0.3 **aB**
Brigitta	2	0.81 ± 0.004 **aA**	0.104 ± 0.008 **aA**	2.35 ± 0.1 **aA**
	10	0.78 ± 0.012 **abA**	0.084 ± 0.007 **abB**	2.41 ± 0.1 **aA**
	50	0.77 ± 0.012 **bA**	0.085 ± 0.001 **abA**	2.31 ± 0.2 **aA**
	250	0.80 ± 0.004 **aA**	0.076 ± 0.004**abAB**	2.62 ± 0.2 **aA**
	500	0.78 ± 0.010 **abA**	0.064 ± 0.005 **bB**	2.86 ± 0.1 **aA**
	1000	0.74 ± 0.005 **bA**	0.069 ± 0.002 **bAB**	2.77 ± 0.24 **aA**
Bluegold	2	0.81 ± 0.016 **aA**	0.116 ± 0.006 **aA**	2.46 ± 0.2 **aA**
	10	0.76 ± 0.013 **bA**	0.117 ± 0.001 **aA**	1.57± 0.2 **bB**
	50	0.77 ± 0.005 **bA**	0.076 ± 0.010 **abA**	1.62 ± 0.2 **bAB**
	250	0.77 ± 0.006 **abB**	0.063 ± 0.003 **bB**	1.64 ± 0.3 **bB**
	500	0.78 ± 0.002 **abA**	0.059 ± 0.003 **bB**	2.38 ± 0.04 **aA**
	1000	0.76 ± 0.004 **bA**	0.056 ± 0.005 **bB**	1.87 ± 0.3 **abB**

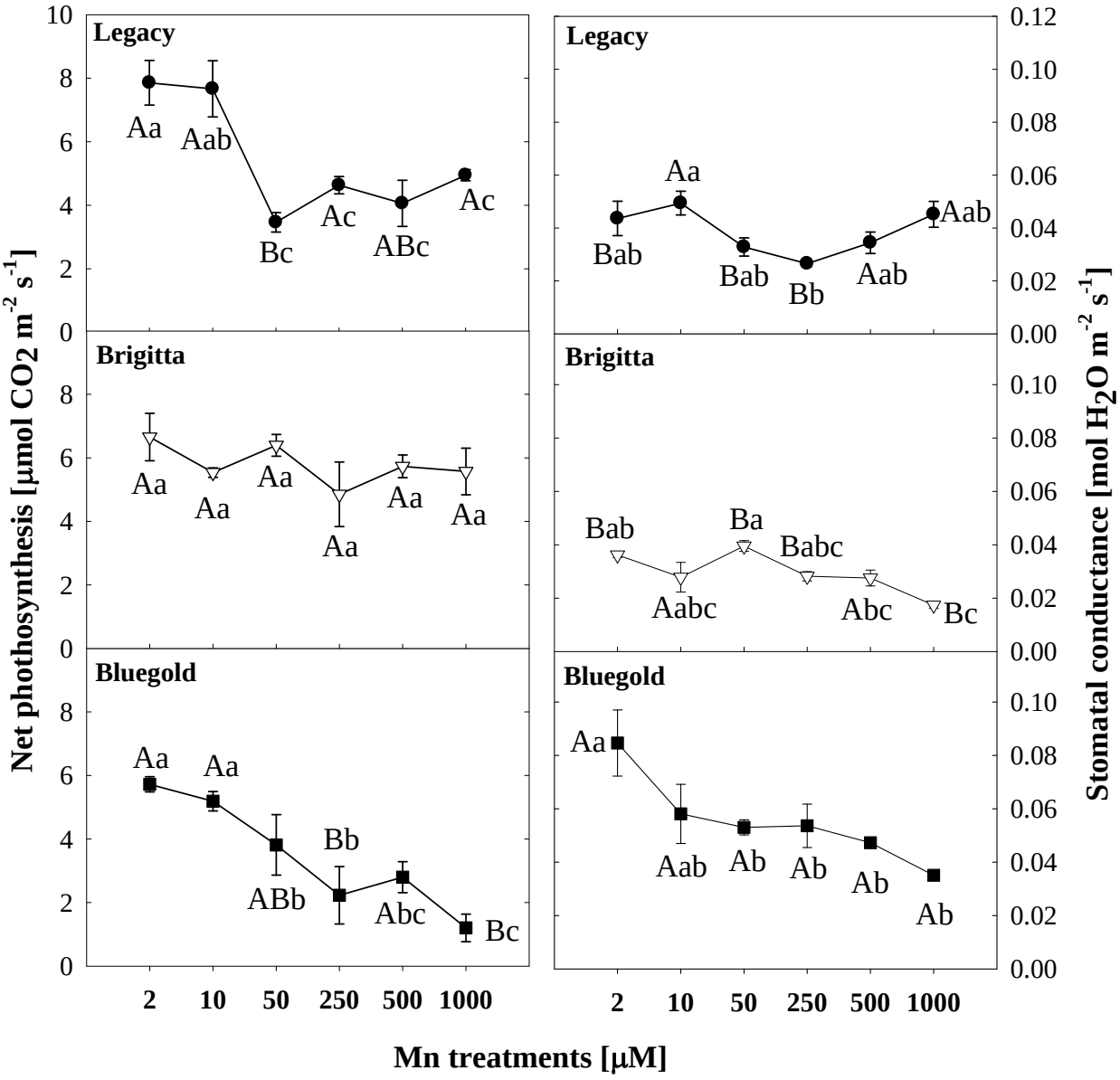

Figure 3. Net photosynthesis (µmol CO_2 m^{-2} s^{-1}) and stomatal conductance (mol H_2O m^{-2} s^{-1}) of three highbush blueberry cultivars subjected to increase Mn treatments at the end of experiment (25 days). Values are means of four measurements. Different lower-case letters indicate significant differences between Mn treatments at the same cultivar. Different upper-case letters indicate significant differences between cultivars at the same Mn treatment ($P \leq 0.05$).

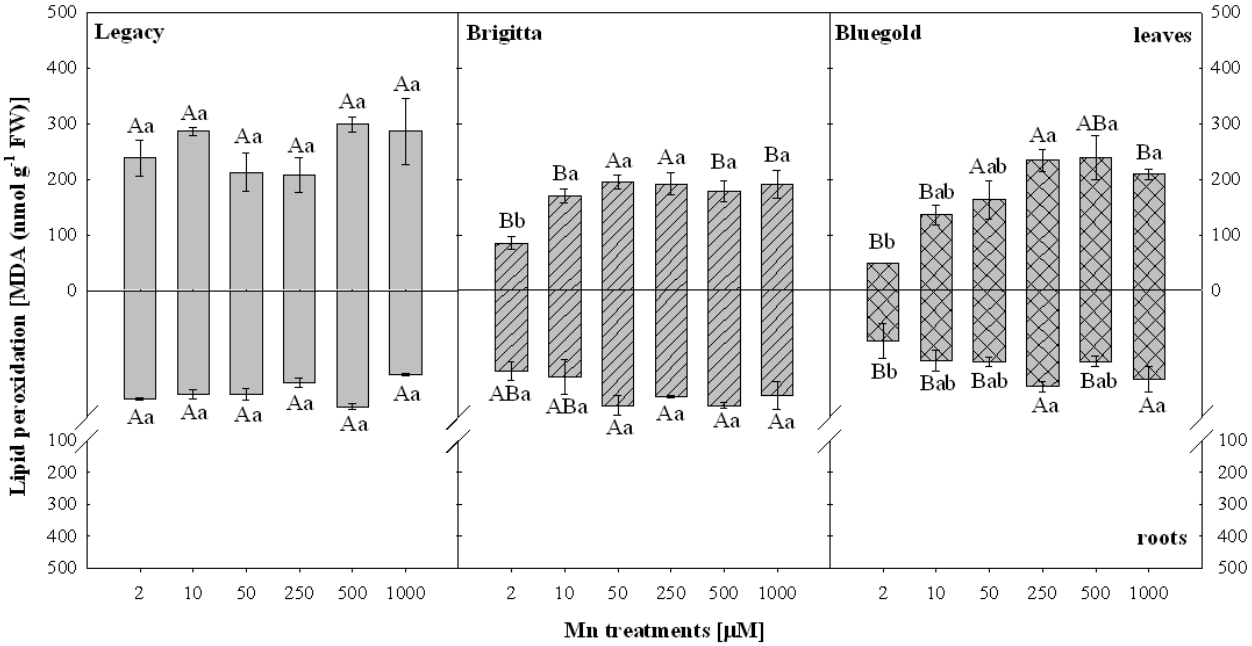

Figure 4. Lipid peroxidation of leaves and roots of three highbush blueberry cultivars subjected to different Mn treatments for 25 days. It was measured as malondialdehyde (MDA). Values are an average of six replicates each ± SE. Different lower-case letters indicate significant differences between Mn treatments at the same cultivar and the same plant organ ($P \leq 0.05$).

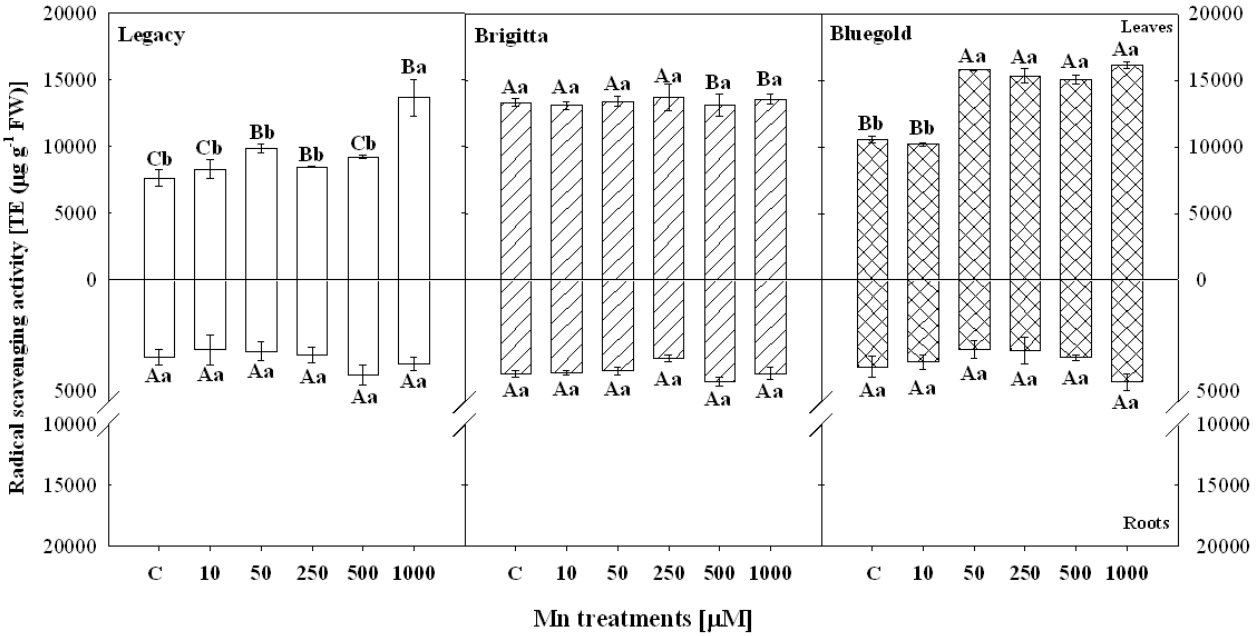

Figure 5. Radical-scavenging capacity (RSA) of three highbush blueberry cultivars subjected to different Mn treatments at leaves and roots at 25 days, measured as Trolox equivalents (TE). Different lower-case letters indicate statistically significant differences between Mn treatments for the same cultivar ($P<0.05$).

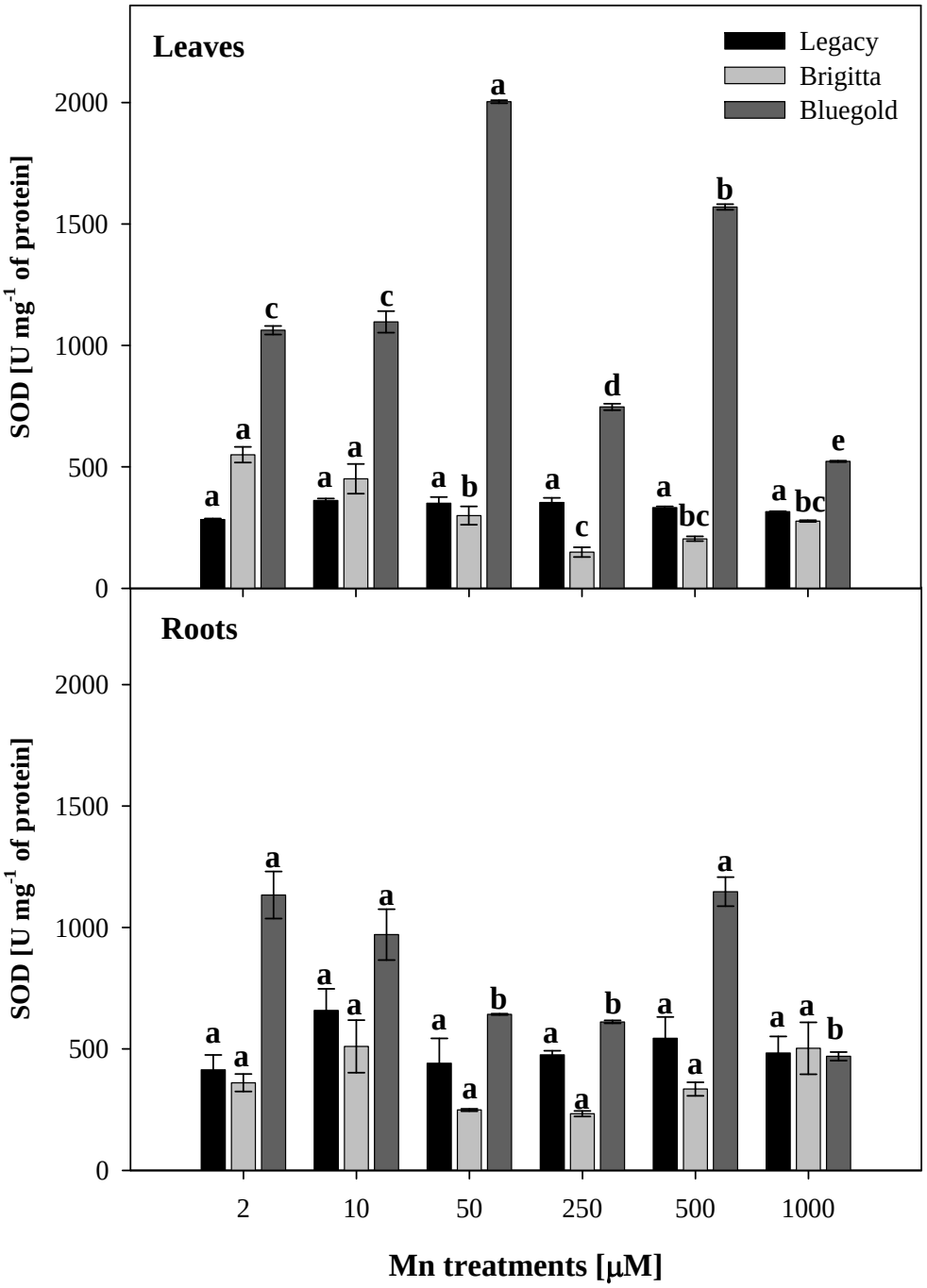

Figure 6. Effect of Mn-excess on the activity of SOD enzyme in leaves and roots of three blueberry cultivars with excess Mn treatments at 25 days of tratment. Values are means of four replicates each ± SE. Different lower-case letters indicate statistically significant differences between Mn treatments for the same cultivar ($P<0.05$).

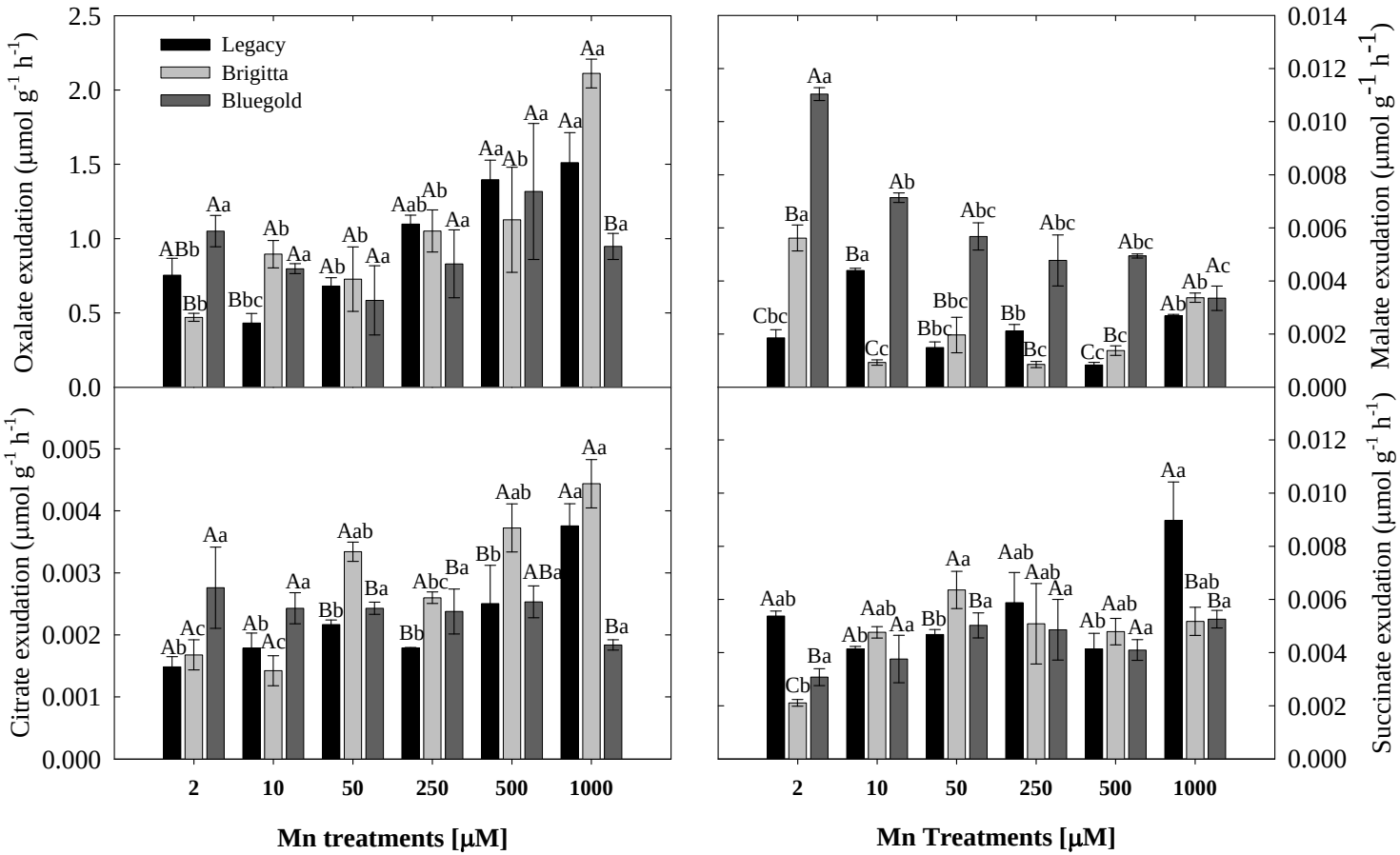

Figure 7. Organic acid anions exuded by roots of blueberry cultivars grown with increasing Mn levels for 25 d. Different lower case letters indicate statistically significant differences between Mn treatments for the same cultivar and different upper case letters show differences between cultivars for the same Mn treatment ($P<0.05$).

Chapter 4:

Excess manganese (Mn) differentially inhibits Photosystem I (PSI) versus Photosystem II (PSII) in *Arabidopsis thaliana.*

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Excess manganese (Mn) differentially inhibits Photosystem I (PSI) versus Photosystem II (PSII) in *Arabidopsis thaliana*

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Abstract

The effects of exposure to increasing Mn concentrations (50 to 1500 μM) from the start of the experiment on the functional performance of photosystem II (PSII) and photosystem I (PSI) and photosynthetic apparatus composition of *Arabidopsis thaliana* were compared. In agreement with earlier studies excess Mn caused minimal changes in the PSII photochemical efficiency measured as Fv/Fm, although the characteristic peak temperature of the $S_{2/3}Q_B$- charge recombinations was shifted to lower temperatures at the highest Mn concentration. SDS-PAGE and immunoblot analyses also did not exhibit any significant change in the relative abundance of PSII-associated polypeptides: PSII reaction center protein D1, Lhcb1 (major light harvesting protein of LHCII complex) and PsbO (OEC33 - 33kDa protein of the oxygen-evolving complex). In addition, the abundance of Rubisco also did not change with Mn treatments. However, plants grown under excess Mn exhibited increased susceptibility to PSII photoinhibition. In contrast, *in vivo* measurements of the redox transients of PSI reaction centre (P700) showed a considerable gradual decrease in the extent of P700 photooxidation ($P700^+$) under increased Mn concentrations compared to control. This was accompanied by a slower rate of $P700^+$ re-reduction indicating a down-regulation of the PSI-dependent cyclic electron flow. The abundance of PSI reaction center polypeptides (PsaA and PsaB) in plants under the highest Mn concentration was also significantly lower compared to the control. The results demonstrate for the first time that PSI is the major target of Mn toxicity within the photosynthetic apparatus of *Arabidopsis* plants. The possible involvement mechanisms of Mn toxicity targeting specifically PSI are discussed.

Keywords: chlorophyll fluorescence, Mn toxicity, photosystem I, PSI- PSII-associated proteins, redox state of P700.

4.1 Introduction

Manganese (Mn) is one of the most abundant metals in the Earth's crust and although it is an important essential micronutrient for all photosynthetic organisms can be also toxic when it is present in excess (Mukhopadhyay and Sharma, 1991; Marschner, 1995). Mn is considered the second most phytotoxic element, after aluminum (Al), affecting negatively the physiological and biochemical properties of plant species (Foy *et al.*, 1978, Foy, 1984; Millaleo *et al.*, 2010). An excess of this metal occurs in acid soils with low pH (<5.5) and/or under reducing conditions (Marschner, 1995; Schaaf *et al.*, 2002), where Mn^{2+} is the predominant solution species and available ion to plant cells (Bradl, 2004). Thus, a Mn excess results in a sharp decrease in shoot height, biomass accumulation and total leaf area of a woody species (*Populus cathayana*) (Lei *et al.*, 2007), a reduction of the dry weights (DW) of both shoots and roots in ryegrass cultivars (*Lolium perenne*) (Mora *et al.*, 2009) and *Trifolium repens* (Rosas *et al.*, 2007). Furthermore, excess Mn can result in oxidative stress as indicated by the accumulation of H_2O_2 (Demirevska-Kepova *et al.*, 2004; Lei *et al.*, 2007), high levels of apoplastic H_2O_2-consuming peroxidases (Fecht-Christoffers *et al.*, 2003) and high level of lipid peroxidation (Mora *et al.*, 2009). Mn-stress induced enhancement of antioxidant enzyme activity in leaves of legumes (González e*t al.*, 1998), it was also demonstrated in perennial ryegrass (Mora *et al.*, 2009) and woody species (Lei *et al.*, 2007). More recently, a proteomic and transcriptomic studies have demonstrated that chloroplastic proteins important for CO_2 fixation and photosynthesis were of lower abundance upon Mn stress of cowpea (Führs *et al.*, 2008).

Mn has an important role in both the structure and functions of the photosynthetic apparatus (Mukhopadhyay and Sharma, 1991). Mn is a constitutive element associated with the oxygen evolving complex (OEC) of photosystem II (PSII), an important multi-protein pigment complex embedded in the thylakoid membranes (Hankamer *et al.*, 1997; Enami *et al.*, 2008). Therefore, the Mn cluster, together with other ions and extrinsic proteins that constitute OEC are required to oxidize water and reduce P680, the reaction center of PSII (Kern and Renger, 2007; Ferreira *et al.*, 2004; Rutherford and Boussac, 2004). In conjunction with photosystem I (PSI) and linear electron transport, these reducing equivalents (electrons) are used primarily in the conversion of CO_2 into carbohydrate (Ferreira *et al.*, 2004). In addition, Mn is indispensable as a cofactor for various enzymes

involved in redox reactions such as Mn-SOD, an essential enzyme involved in protection against oxidative stress in plants (Burnell, 1988; Bowler *et al.*, 1994).

A number of studies have suggested that chloroplasts and photosynthesis are the major targets of Mn toxicity. Indeed, increased amounts of Mn were reported for chloroplasts isolated from Mn-stressed common bean (González and Lynch, 1999) and rice leaves (Lidon *et al.*, 2004). Distinctive ultrastructural changes showing swelling of granal and stromal thylakoids have been also observed in the chloroplasts of *Citrus volkameriana* (Papadakis *et al.*, 2007) and maize plants (Doncheva *et al.*, 2009) under Mn excess. It has been demonstrated that high Mn accumulation is associated with inhibition of the net photosynthesis and carboxylation efficiency in various plant species. The decline of photosynthesis is considered as one of the major mechanisms constituting the toxic effects of excess Mn and is proposed as an early indicator for Mn toxicity in tobacco (Nable *et al.*, 1988), rice (Lidon *et al.*, 2004) and wheat (Macfie and Taylor, 1992). Reduced CO_2 assimilation induced by excess Mn was also reported for common bean (González and Lynch, 1997), deciduous broad leaved trees (Kitao *et al.*, 1997*a*) and seedlings of *Citrus grandis* (Li *et al.*, 2010). Interestingly, the maximum efficiency of PSII photochemistry (F_v/F_m) was not substantially affected by Mn accumulation in various plant species over a wide range of leaf Mn concentrations (Nable *et al.*, 1988; Kitao *et al.*, 1997b; Subrahmanyam and Rathore, 2000; Hajiboland and Hasani, 2007; Doncheva *et al.*, 2009).

The reduction in photosynthesis by excess leaf Mn has been generally attributed to modification of ribulose-1,5- bisphosphate carboxylase/oxygenase (Ohki, 1984; Houtz *et al.*, 1988; McDaniel and Toman, 1994; Kitao *et al.*, 1997*b*). It has been demonstrated that a high level of Mn affects primarily the activity rather than the amount of RUBISCO (Houtz *et al.*, 1988; Chatterjee *et al.*, 1994) and the presence of excess Mn induces enhanced oxygenase activity (Jordan and Ogren, 1983). In addition, the decline of photosynthesis under Mn stress conditions was also ascribed to peroxidative impairment of photosynthetic enzyme activities caused by polyphenol oxidation products (Panda *et al.*, 1987; Vaughn and Duke, 1984).

In spite of these studies the mechanisms of Mn toxicity causing a decrease in CO_2 assimilation are still not well understood. In addition, very limited information concerning the toxic effect(s) of excess Mn on the polypeptide composition of both PSII and especially

PSI is available. Therefore, the objective of this study was to evaluate the role of specific Mn induced changes in the structure and function of PSII and PSI, which could help to understand the mechanisms by which Mn-excess may cause a decrease of CO_2 assimilation in *Arabidopsis thaliana.*

4.2 Materials and methods

4.2.1 Plant material and growth conditions. Seeds of *Arabidopsis thaliana* (wild type Columbia) were germinated in a substrate mix (82.5% sphagnum peat moss, 12.5% perlite, 5% vermiculite-Pro-Mix, Premier Tech Horticulture Ltd.) in controlled environment growth cabinets (Model GCW15, Environmental Growth Chambers, Chagrin Falls, OH) with a photosynthetical active radiation (PAR) of 250 µmol photons m^{-2} s^{-1}, 20/20°C day/night temperatures, 50% relative humidity and an 8-h photoperiod to prevent flowering. Water was supplied every five days. After 15 days, seedlings were transplanted separately in pots with vermiculite and placed in trays. Each tray containing seven pots (one plant per pot), were supplied with Hoagland nutrient solution for two weeks before applying the Mn treatments (see below).

4.2.2 Manganese treatments. Manganese treatments included the final concentrations: 18 (control), 50, 500, 1000 and 1500 µM Mn. Doses applied were prepared according to Delhaize *et al*., (2007). Manganese was applied as $MnCl_2$ x $4H_2O$. Control plants exposed to 18 µM Mn was optimal dose for Mn for *Arabidopsis* (Cailliatte *et al.*, 2010). The five Mn treatments were grown in five labeled trays, with 500 mL of Hoagland's solution. The trays were maintained in controlled environment growth chambers under the same conditions described above. The pH was adjusted to 5.3 with diluted HCl daily and nutrient solution was changed every five days. Plants were subjected to these treatments for 21 days before harvest.

4.2.3 Plant growth measurements. Prior to beginning the Mn-treatments, three plant samples were dried in a forced-air oven (70°C, 48 hrs) and weighed to determine dry weight (W1) at 0 day. Similarly, at the end of the experiment, plants were harvested and

collected for fresh and dry weight measurements (W2). These data were used to determine mean relative growth rate (RGR) according to Fernando *et al.* (2009):

$$RGR = \frac{(\ln W2 - \ln W1)}{(t2 - t1)}$$

as g g^{-1} d^{-1} DW.

4.2.4 Manganese concentration. For Mn chemical analysis, samples of shoot and roots were dry-ashed in a muffle furnace at 500°C for 8 h and digested with 2 M HCl. Manganese was extracted as described by Sadzawka *et al.* (2004), and the Mn concentration was determined using a simultaneous multi-element atomic absorption spectrophotometer (Model 969 atomic absorption spectrometer; Unicam, Cambridge, UK).

4.2.5 Thylakoid preparation, SDS-PAGE and immunoblotting. Thylakoid membranes for SDS-PAGE were isolated as described earlier (Krol *et al.*, 1999). Leaf material was ground in cold isolation buffer (50 mM Tricine, 0.4 M sorbitol, 10mM NaCl, 5mM $MgCl_2$, pH 7.8) in a mortar on ice, filtered through two layers of miracloth (typical pore size 22-25 µm; Calbiochem), and centrifuged for 15 min (10,000 g). The supernatant was removed and the pellet was resuspended in cold isolation buffer. Total chlorophyll concentration was measured in 90% (v/v) acetone (Arnon, 1949). For immunodetection of Rubisco total leaf proteins were extracted as described in Rosso *et al.* (2009). Protein content was measured using a BCA protein assay kit (Pierce) by following the absorbance at 562 nm using a Beckman DU-640 spectrophotometer (Beckman Coulter). Proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (1970), using 15% (w/v) polyacrylamide gel in the presence of 6 M urea in the separating gel. Chloroplast thylakoids were solubilized with SDS (SDS: chlorophyll, 20:1) and 15 µg of chlorophyll was loaded per lane. All samples for separation of total proteins were loaded on an equal protein basis of 20 µg protein per lane (Rosso *et al.* 2009). Immunoblotting was performed by electrophoretically transferring the proteins from SDS-PAGE gel to nitrocellulose membrane (Bio-Rad) according to the method of Towbin *et al.* (1979). Proteins were probed with antibodies (AgriSera AB, Vanas, Sweden) raised against the reaction center polypeptides of PSI: PsaA, PsaB (1:2000), the major light harvesting

protein of PSII complex (LHCII): Lhcb1 protein, (1:5000), the PSII oxygen evolving complex (OEC) extrinsic protein: PsbO (33 kDa) (1:2000), the PSII reaction center protein: D1 and ribulose-1.5-bisphosphate carboxylase/oxigenase protein (Rubisco) (1:5000). As secondary antibodies goat anti-rabbit IgG conjugated with horse-radish peroxidase (Sigma-Aldrich) were used. Polypeptides were detected using enhanced chemiluminescence (ECL) detection kit (Amersham Biosciences) and visualized by exposing the membrane to x-ray film. Densitometric scanning and analysis of X-ray films from each replicate immunoblot was performed with a Hewlett Packard ScanJet 4200C desktop scanner and ImageJ 1.41o densitometry software (Wayne Rosband, National Institute of Health, USA, http://rsb.info.nih.gov.ij).

4.2.6 Measurement of the redox state of P_{700}. The redox state of P_{700} was determined *in vivo,* in dark adapted (20 min) *Arabidopsis* leaves under growth temperature and ambient O_2 and CO_2 conditions using a PAM-101 modulated fluorometer equipped with a dual-wavelength emitter-detector ED-P700DW unit and PAM-102 units (Klughammer and Schreiber, 1991) as described in detail by Ivanov *et al.* (1998). Far-red light (λ_{max}=715 nm, 10 W m^{-2}, Schott filter RG 715) was provided by an FL-101 light source. The redox state of P700 was evaluated as the absorbance change around 820nm ($\Delta A_{820–860}$) in a custom-designed cuvette. Multiple turnover (MT, 50 ms) and single turnover (ST, half peak 14 µs) saturating flashes were applied with XMT-103 and XST-103 (Walz) power/control units, respectively. The relative functional pool size of intersystem electrons on a P_{700} reaction center basis was calculated as the complementary area between the oxidation curve of P700 after either ST or MT pulse excitation (ST- and MT-areas) and the stationary level of P700 under FR excitation (Asada *et al.*, 1993; Ivanov *et al.*, 1998). Capacity of PSI cyclic electron (e^-) flow was determined as the half time for the dark decay of the P700 signal (Ivanov *et al.*, 1998).

4.2.7 Modulated chlorophyll fluorescence measurements. Modulated imaging fluorometer (IMAGING-PAM, Heinz Walz GmbH, Efeltrich, Germany) was used for capturing the chlorophyll fluorescence images and estimation of the maximal photochemical efficiency of PSII [$F_v/F_m = (F_m - F_o)/F_m$], quantum yield of PSII

photochemistry (Φ_{PSII}), photochemical (qP) and non-photochemical (NPQ) fluorescence quenching parameters using the nomenclature of van Kooten and Snel (1990) as described earlier (Ivanov *et al.*, 2006a). Control and Mn-treated *Arabidopsis* plants were dark adapted (20 min) and all chlorophyll fluorescence measurements were performed *in vivo* at room temperature. Fluorescence images were captured by a CCD camera (IMAG-K, Allied Vision Technologies) featuring 640x480 pixel CCD chip size and CCTV camera lens (Cosmicar/Pentax F1.2, f=12 mm). Light emitting diode ring array (IMAG-L) consisting of 96 blue LEDs (470 nm) provided standard modulated excitation intensity of 0.5 μmol quanta m^{-2} s^{-1} (modulation frequency 1-8 Hz) for measuring the basal (F_0) chlorophyll fluorescence and a saturation pulse of 2400 μmol quanta m^{-2} s^{-1} PAR for measuring the maximal chlorophyll fluorescence (F_m). The maximal PSII photochemical efficiency (F_v/F_m) was determined as ($F_m - F_0$)/ Fm. The effective photochemical efficiency of PSII (Φ_{PSII}) was calculated from the expression (F'_m–F_s)/F'_m (Genty *et al.*, 1989), photochemical quenching (qP) was calculated as: (F'_m-F_s)/(F'_m-F'_0) (Schreiber *et al.*, 1994), electron transport rate (ETR) was calculated as: ETR = PAR x 0.5 x·ΦPSII x 0.84 (Genty *et al.*, 1989), regulated non-photochemical quenching (Φ_{NPQ}), constitutive photochemical quenching (Φ_{NO}) was determined according to Kramer et al. (2004). All measurements were performed at 0, 5, 10, 15 and 21 days in plants subjected to the different Mn treatments.

At the end of experiments (21 days), leaves of *Arabidopsis* plants growing under the different Mn treatments and 250 μmol m^{-2} s^{-1} PAR (normal light), were cut and exposed to high light (1000 μmol s^{-1} m^{-2}, PAR) at 1, 2 and 4 hours. During each time of exposition, F_v/F_m was determined.

4.2.8 Thermoluminescence measurements. Thermoluminescence (TL) measurements of control and Mn-treated *Arabidopsis* leaves were performed on a personal-computer-based TL data acquisition and analysis system as described earlier (Ivanov *et al.*, 2001; 2006b). A photomultiplier tube (Hamamatsu R943–02, Hamamatsu Photonics K.K., Shizuoka-ken, Japan) equipped with a photomultiplier power supply (Model PS-302, EG&G Electro Optics) and a preamplifier (Model C1556-03) was used as a radiation measuring set. The heating rate was 0.6°C s^{-1}. For identifying the $S_{2/3}Q_B^-$ charge recombination peaks, dark

adapted leaf discs were subjected to two consecutive saturating microsecond flashes of white light (1.5 μs peak width at 50% of maximum) applied by a xenon-discharge flash lamp (XST103, Heinz Walz GmbH, Effeltrich, Germany). Dark adapted leaves (20 minutes at 20°C) were cooled to 2°C prior to exposing to the flashes. The nomenclature of Sane *et al.* (2012) was used for characterisation of the TL glow peaks.

4.2.9 Experimental design and statistical analysis. The experimental design is a randomized split design with one species x·five Mn treatments x·seven replicates for the physiological determinations, and one species x five Mn treatments x three replicates x four times (0, 1, 2 and 4 hours) for the photoinhibition measurements. Data correspond to the means of replicates for each determination as indicated above. Data were tested in their normality and equal variance by the Shapiro-Wilk test and then data were analyzed by a one way ANOVA. Significant differences among means were established by using the multiple comparisons test of Tukey's ($P < 0.05$). All analyses were performed with Sigma Stat 2.0 software (SPSS, Chicago), where differences between the values were considered significant at $P \leq 0.05$.

4.3 Results

A statistically significant decrease in total biomass of leaves treated with 500 and 1500 μM Mn concentrations was found compared to the control plants (18 μM Mn), although a decrease of the total leaf biomass was found at all Mn concentrations used. In contrast, roots biomass exhibited significant decrease in all treatments ($P<0.05$) (Fig. 1). It should be noted, however, that while the decrease of leaf biomass was only 25% lower at the highest Mn concentration used (1500 μM), the biomass of roots was more affected and demonstrated 2.5-fold decrease even at the lowest Mn concentration tested (50 μM). This is consistent with the relative growth rate (RGR) data, where a considerably higher reduction of root RGR was observed compared to leave across the Mn treatments relative to control plants ($P<0.05$) (Table 1). In addition, visual symptoms of Mn toxicity (chlorosis) in leaves were observed predominantly in the highest Mn- treatment (data not shown).

Analysis of the total Mn amount in plants exposed to increasing Mn concentrations have demonstrated a gradual increase of Mn in leaves and roots (Fig. 2). However, while Mn

concentrations of roots reached values up to ~8100 mg kg^{-1} Mn at the highest Mn-treatment, which represent a 40-fold increase of Mn, the increased accumulation of Mn in leaves was much lower (15-fold) compared to roots (Fig. 2). Thus, the differential effects of Mn treatments on the total biomass and RGR in leaves and roots could be explained by the higher accumulation of Mn in roots.

The effects of exposure to increasing Mn concentrations on the photochemical efficiency of PSII measured as F_v/F_m in *Arabidopsis* leaves are presented in Table 2. A time course measurements did not reveal any statistically significant changes of F_v/F_m values at the lower Mn doses (50 μM and 500 μM) for the entire period of treatment compared to control plants (18 μM Mn). At higher Mn concentrations (1000 μM and 1500 μM) a small reduction (6%) of F_v/F_m was observed only at a later stage (days 15 and 21) of treatment, the statistically significant ($P<0.05$) differences being registered at the harvest time (21 days) (Table 2). Moreover, minimal Mn-induced changes were detected in the light response curves of photochemical quenching (qP), effective quantum yield of PSII (Φ_{PSII}) and PSII electron transport rates (ETR) even after 21 days of Mn-treatments with the highest dose tested (1500 μM) (data not shown).

In addition, thermoluminescence (TL) measurements were used as an alternative approach for assessing the effects of Mn-excess on the photosynthetic PSII-associated electron transfer reactions especially at its reducing side (Vass and Govindjee, 1996; Sane *et al.*, 2012). Since most of the photosynthetic TL components have been assigned to arise from the reversal of light-driven charge separation in PSII, TL properties of photosynthetic apparatus provide information on the activation energies associated with the back reactions of electron acceptors (Q_A and Q_B) with the electron donors (S_2 and S_3) of PSII (Vass and Govindjee, 1996; Sane, 2004; Sane *et al.*, 2012). The temperature maxima (T_M) of the TL peaks related to the recombination of these charge pairs reflect the activation energies and hence a measure of the redox potentials of the participating oxidized and reduced donors (de Vault and Govindjee, 1990). Typical TL glow curves representing S_2/S_3Q_B- charge recombinations of control and Mn-treated *Arabidopsis* plants obtained following excitation with two consecutive saturating flashes are shown in Fig. 3. The experimental data summarized in Table 3 indicate that treatment with a Mn dose of 500 μM did not exhibit significant differences of the TL peak position, while treatment with the highest

concentration (1500 μM) induced a low temperature shift of the T_M to 25.8°C compared to control plants. Besides this, the amplitudes and the integrated areas of the TL peaks representing the $S_2/S_3Q_B^-$ charge recombination used for assessing the PSII photochemistry was not affected at 500 μM Mn-excess, but the overall TL yield was significantly reduced (45%) in plants treated with 1500 μM Mn compared to controls (Table 3).

To further test whether this small but significant Mn-induced effect on PSII photochemistry had physiological implications under additional stress conditions, *Arabidopsis* leaves subjected to different Mn treatments for 21 days were exposed to high light stress (Table 4). Indeed, the photoinhibitory effect on PSII, measured as a decrease in F_v/F_m, was much stronger (49%) at the highest Mn treatment (1500 μM) compared to control plants and 500 μM Mn treated plants exhibited only a 15% decrease in F_v/F_m values after 4 h of exposure to high light (Table 4).

The extent of far-red light-induced absorbance decrease at 820 nm ($\Delta A_{820-860}$) of *Arabidopsis* leaves (Klughammer and Schreiber, 1991; Ivanov *et al.*, 1998; Ivanov *et al.*, 2006a) was used to estimate the potential functional differences of PSI and photosynthetic electron transport pathways between plants exposed to different Mn treatments. Typical traces representing *in vivo* measurements of oxidation-reduction transients of P_{700} in control and Mn-treated plants are shown in Fig. 4. The relative amount of $P700^+$, measured as $\Delta A_{820-860}$, gradually decreased with increasing Mn concentrations and was 30% lower in Mn-treated plants at the highest concentration used (1500 μM) (Fig. 4, Table 5). Concomitantly, kinetic measurements of dark re-reduction of $P700^+$ after turning off the far-red light, which is thought to reflect the extent and/or capacity for cyclic electron flow around PSI (Maxwell and Biggins, 1976; Ravenel *et al.*, 1994), indicated significantly slower (46%) re-reduction of $P700^+$ in Mn(1500 μM)-treated compared to control plants (Table 5). In addition, the apparent electron donor pool size to PSI (e^- /P700) estimated by measuring single-and multiple-turnover flash-induced $\Delta A_{820-860}$ under steady-state oxidation of PSI by far-red light (Asada *et al.*, 1993; Ivanov *et al.*, 1998) demonstrated a significant decrease in Mn-treated plants (Table 5). This indicates that the pool size of electrons that can be donated to photo-oxided P700 ($P700^+$) from the stroma in control plants was 37% higher compared to plants treated with the highest Mn dose (13 electrons per P700) (Table 4).

The major photosynthetic components within the thylakoid membranes of control and Mn-treated *Arabidopsis* plants were compared by SDS-PAGE and immunodetection to quantify their relative abundance. Immunoblot analyses did not exhibit any significant Mn-stress induced changes in the relative abundance of PSII-associated polypeptides, as revealed by the densitometry analysis of the immunoblot bands for D1 (the PSII reaction center protein), Lhcb1 (major light harvesting protein of LHCII complex) and PsbO (extrinsic protein of the oxygen-evolving complex (OEC) (Fig. 5A, B). The abundance of Rubisco was only marginally affected by the Mn treatments. In contrast, the abundance of reaction center polypeptides of PSI (PsaA and PsaB) was significantly reduced in Mn-treated compared with control thylakoids (Fig. 5A). The densitometric analysis demonstrated that the relative abundance of PsaA and PsaB in Mn-treated *Arabidopsis* was only about 20% and 60%, respectively of that observed in the control plants (Fig. 5B). Thus, the quantitative analysis of photosynthetic polypeptides clearly indicates that excess Mn has a greater effect on PSI-associated proteins rather than PSII. Consequently, the differences in the extent of P700 oxidation ($P700^+$) and the kinetics of $P700^+$ reduction between control and Mn-treated plants are consistent with the lower levels of PSI-associated immundetectable PsaA and PsaB protein complexes.

4.4 Discussion

In agreement with a number of previous studies examining the effects of Mn stress on various plant species (Mora *et al.*, 2009; Alam *et al.*, 2006; Lei *et al.*, 2007; Doncheva *et al.*, 2009; Stoyanova *et al.*, 2009; Khabaz-Saberi *et al.*, 2010) *Arabidopsis* plants subjected to increasing Mn concentrations, also exhibited a reduction in dry weight of both shoots and roots at doses between 500 and 1500 µM Mn (Fig. 1). The decline in biomass corresponded with a gradual increase of Mn concentrations in both shoot and roots of *Arabidopsis* subjected to excess Mn supply (Fig. 2). It should be noted that the decline of biomass was more pronounced in roots, where even at the lowest Mn treatment (50 µM) the dry weight was 2.5 lower compared to leaves. Similar results have been reported by Delhaize *et al.* (2007), where transporter proteins were implicated in the endogenous Mn tolerance of wild-type *Arabidopsis*. More recently, Mora *et al.* (2009) demonstrated that Mn-tolerant ryegrass cultivars accumulated higher Mn concentrations in roots than shoots,

while Mn sensitive cultivars exhibited a greater Mn translocation from roots to shoots. These results are also consistent with studies in legumes such as white clover (*Trifolium repens* L.) (Rosas *et al.*, 2007). However, in two contrasting populations of *Populus cathayana*, acclimated to wet and dry climate exposure to excess Mn caused an increase in Mn content of plant tissues especially in leaves and a visual symptoms of Mn toxicity (chlorosis) at high Mn concentrations (Lei *et al.*, 2007). The chlorosis observed in our experiments (data not shown) also correspond to a decreased amounts of both Chl *a* and Chl *b* could be due to a higher Mn accumulation in leaves after exposure to Mn excess, thus suggesting a damage to the photosynthetic apparatus as reported by Demirevska-Kepova *et al.* (2004).

Interestingly, while reduced CO_2 assimilation induced by excess Mn has been reported in many species and is considered one of the major physiological effects of Mn toxicity (Nable *et al.*, 1988; González and Lynch, 1997; Kitao *et al.*, l997*a*; Lidon *et al.*, 2004; Macfie and Taylor, 1992; Li *et al.*, 2010), the functional integrity of the photosynthetic apparatus assessed by the maximum efficiency of PSII photochemistry (F_v/F_m) did not decline as a result of exposure to excess Mn in various plant species (Nable *et al.*, 1988; Kitao *et al.*, 1997b; Subrahmanyam and Rathore, 2000; Hajiboland and Hasani, 2007; Doncheva *et al.*, 2009). However, some studies have reported a substantial decrease in F_v/F_m as a result of excess Mn treatment in *Citrus* species (Papadakis *et al.*, 2007; Li *et al.*, 2010), rice (Lidon *et al.*, 2004), Mn-sensitive maize (Doncheva *et al.*, 2009) and cucumber (Feng *et al.*, 2009). Furthermore, Kitao *et al.* (1997*b*) have shown that the potential maximum efficiency of PSII photochemistry (F_v/F_m) is not affected by excess Mn in white birch, although the reduction state of PSII primary electron acceptor (Q_A) was increased at high Mn concentrations. These results clearly indicate that the excess Mn- induced decline in CO_2 assimilation may or may not be accompanied by changes in PSII photochemistry and this response is species dependent. The experimental data presented in this study also failed to demonstrate any substantial effects of excess Mn within a wide range of Mn concentrations on the maximum efficiency of PSII photochemistry in *Arabidopsis* plants (Table 2). Assessing the relative abundance of PSII-associated proteins also showed no changes in the immuno-detectable amounts of PSII-reaction center protein D1, the light harvesting chlorophyll-protein complex of PSII (Lhcb1) and the manganese stabilizing

33kDa-protein of the water splitting complex of PSII (PsbO) polypeptides (Fig. 5) in plants exposed to high Mn concentrations compared to control *Arabidopsis*. However, the observed low temperature shift of the $S_{2,3}Q_B^-$ charge recombination and much lower overall TL emission implies lower redox potential of Q_B (Fig. 3; Table 3) confirms the suggestion of altered reduction state of PSII acceptor side in Mn-stressed plants (Kitao *et al.*, 1997*b*). Since Q_A is in quasi-equilibrium with Q_B and the PQ pool, our results imply that lowering the redox potential of Q_B will decrease the probability for forward electron transfer between the two quinone acceptors by shifting the redox equilibrium between $Q_A^-Q_B$ and $Q_AQ_B^-$ towards $Q_A^-Q_B$ (Minagawa *et al.*, 1999; Ivanov *et al.*, 2002; 2003) in plants exposed to high Mn concentrations.

In addition to the lack of significant inhibitory effects of excess Mn on PSII photochemistry discussed above, an earlier study reported that the photochemistry of photosystem I (PSI) and the photosynthetic electron transport were not significantly affected during early development of Mn toxicity in tobacco plants (Nable *et al.*, 1988). However, a decreased Hill activity in isolated chloroplasts was found in mungbean leaves exposed to toxic Mn concentrations (Sinha *et al.*, 2002). More recently, Li *et al.* (2010) have suggested that Mn excess can effectively impair the whole photosynthetic electron transport chain, thus restricting the production of reducing equivalents and limiting the rate of CO_2 assimilation in *Citrus grandis* seedlings. Despite these few studies, the potential effect(s) of excess Mn on the functional/structural integrity of PSI remains elusive. To our knowledge, the results presented in this study are the first report of an *in vivo* assessment of high Mn concentrations on PSI photochemistry. In contrast to PSII photochemistry, *in vivo* measurements of the oxidation state of P700($P700^+$) (Klughammer and Schreiber, 1991; Ivanov *et al.*, 1998), the primary donor of PSI demonstrated that the relative amount of oxidizable P700($P700^+$) decreased by 30% in Mn-treated *Arabidopsis* plants at concentrations above 1000 μM (Fig. 4, Table 5). The functional impairment of PSI photochemistry by excess Mn was accompanied by a significant reduction in the abundance of PSI reaction center polypeptides (PsaA and PsaB) (Fig. 5). This clearly indicates that the major target of Mn toxicity within the photosynthetic electron transport chain of *Arabidopsis* is PSI rather than PSII related components. The reduced amounts of PSI reaction center polypeptides PsaA and PsaB would imply acceptor side limitations of the

photosynthetic electron transport and this could explain the increased reduction state Q_A in Mn-stressed plants reported earlier (Kitao *et al.*, 1997*b*). Moreover, decreased expression of another Fe-containing chloroplastic protein precursor, ferredoxin-1 serving as a terminal electron acceptor of the photosynthetic electron transport observed in Mn-treated young rice leaves also supports Mn-induced limitations at the acceptor side of PSI (Führs *et al.*, 2010).

One of the major mechanisms considered for Mn toxicity involves the inhibition of other essential cations including Fe, thus suggesting that a Mn-induced Fe-deficiency may play a key role in the physiological responses to excess Mn (Foy *et al.*, 1978; Foy, 1984; Kohno *et al.*, 1984). More recently, chloroplast alterations in maize plants exposed to excess Mn (Doncheva *et al.*, 2009) and Mn-toxicity in young rice leaves (Führs *et al.*, 2010) have been also ascribed to Mn-induced Fe deficiency rather than to direct Mn-induced oxidative stress. Given that about 80% of the plant Fe is located in the chloroplast (Terry and Abadia, 1986) and the functional photosynthetic apparatus requires 22-23 iron atoms, of which PSI is the most Fe-abundant component (Ferreira and Straus, 1994), it seems reasonable to assume that the observed lower abundance of PSI reaction center polypeptides and the associated decline of PSI photochemistry in Mn-treated *Arabidopsis* plants were consequences of a Mn-induced moderate Fe deficiency.

Although light energy is important for photosynthetic processes in plants, an excess of light can be also harmful because it can result in photoinhibition, which can be exacerbated when it is combined with other stresses (Powles, 1984; Aro *et al.*, 1993; Sonoike, 1996). Photoinhibition is a complex phenomenon that may cause damage to the photosynthetic apparatus reducing the photosynthetic efficiency when light conditions exceed the photon requirements for photosynthesis (Murata *et al.*, 2007). It is considered that PSII is the main site of photoinhibition (Aro *et al.*, 1993; Sonoike, 1996; Takahashi and Murata, 2008), being more unstable than PSI, because the D1 protein, one of the two major heterodimeric polypeptides of the PSII reaction center complex, has a very high, light-dependent turnover rate (Scheller and Haldrup, 2005; Aro *et al.*, 1993; Burnap, 2004; Takahashi and Murata, 2008). Earlier reports have shown that the susceptibility to Mn toxicity is strongly dependent on the light intensity and exposure of Mn-treated plants to high light can exacerbate the toxic effect of Mn. (Nable *et al.*, 1988; Horiguchi, 1988; González *et al.*,

1998; Clair and Lynch, 2004; Hajiboland and Hasani, 2007). Our results also demonstrate that *Arabidopsis* plants pre-disposed to high Mn concentrations are more susceptible to photoinhibitory damage of PSII photochemistry in a concentration dependent manner (Table 4).

Apart from the radiation less dissipation of excess excitation energy in the chlorophyll pigment bed of LHCII, associated with the formation of the xanthophylls pigment zeaxanthin (Z), which is considered one of the major protective mechanisms against photoinhibitory damage (Horton *et al.*, 1996; Niyogi, 1999), PSI-dependent cyclic electron transport (CET) has been also suggested to play a significant role in preventing the photoinhibitory damage of the photosynthetic apparatus during exposure of plants to high light conditions (Munekage *et al.*, 2002; Takahashi *et al.*, 2009). Considering the increased re-reduction rate of $P700^+$ in *Arabidopsis* (Table 5), we believe that the higher susceptibility of plants exposed to excess Mn to photoinhibition (Table 4) might be due to lower capacity of PSI-driven cyclic electron flow under conditions of Mn toxicity.

In summary, the results presented in this research demonstrate for the first time that exposure of *Arabidopsis* plants to excess Mn causes specific negative effects on the abundance of polypeptides comprising the reaction center of PSI, thus resulting in decreased PSI photochemistry and lower capacity for CET, which may be due to a Mn-induced Fe-deficiency and may have critical physiological implications under conditions of Mn toxicity in higher plants.

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Table 1. Relative growth rates (RGR) of *Arabidopsis thaliana* (leaves and roots) growing at increased Mn treatments under acidic conditions (pH 5.3) for 21 d in comparison with the control. Different lower-case letters indicate statistically significant differences between the Mn treatments ($P < 0.05$).

Relative growth rate (RGR) ($g\ g^{-1}\ d^{-1}$ DW)		
Mn treatments	Leaves	Roots
C	0.069 ± 0.001 a	0.206 ± 0.006 a
50 µM	0.068 ± 0.003 a	0.071 ± 0.005 b
500 µM	0.046 ± 0.006 b	0.053 ± 0.006 b
1000 µM	0.059 ± 0.003 ab	0.064 ± 0.007 b
1500 µM	0.040 ± 0.004 b	0.057 ± 0.004 b

Table 2. Maximal photochemical efficiency of PSII (Fv/Fm) in leaves of *Arabidopsis thaliana* measured at 0, 5, 10, 15 and 21 days subjected to increasing Mn treatments (µM). Results are means of 5 repetitions of two plants each ± SE. Different lower case indicate significant differences between the Mn treatments at the same time, while different upper-case letters indicate significant differences between the times at a same Mn treatment ($P < 0.05$).

Fv/Fm					
Mn treatments (µM)	**Time (days)**				
	0	5	10	15	21
C	0.803 aA	0.803 aA	0.803 aA	0.803 aA	0.803 aA
50	0.806 aA	0.799 aA	0.800 aA	0.800 aA	0.800 aA
500	0.803 aA	0.801 aA	0.804 aA	0.787 bA	0.791 aA
1000	0.797 aA	0.805 aA	0.800 aA	0.778 bB	0.773 bB
1500	0.802 aA	0.798 aA	0.775 bB	0.769 bcBC	0.756 cC

Table 3. Characteristic thermoluminescence (TL) peak emission temperatures (T_M) and the overall TL emission area (A) of S_2/S_3Q_B- glow peaks of control and Mn-treated (21 days) *Arabidopsis* plants. The samples (leaf disks) were dark adapted for 30 min then cooled to 2°C and subsequently illuminated with two single turnover flashes of white light. The peak areas are presented as a percentage of the total TL light emission in control leaves. Mean values ± SE were calculated from 4 independent experiments.

Mn treatments (µM)	T_M **(°C)**	**A (%)**
Control	28.7 ± 1.1	100.0
500 µM	29.1 ± 1.3	106.2 ± 7.6
1500 µM	25.8 ± 0.9	54.5 ± 7.9

Table 4. Effect of high light treatments (1000 µmol photons m^{-2} s^{-1}, PFD, for 1, 2 and 4 hours) on the maximal photochemical efficiency of PSII measured as Fv/Fm in control *Arabidopsis thaliana* leaves and plants exposed for 21 days to different Mn doses. Results are means of 5 repetitions in three leaves of two plants each ± SE. Time 0 was measured in plants subjected to 250 µmol m^{-2} s^{-1} PFD. Different upper-case letters indicate significant differences between the times of exposure at the same Mn treatment ($P < 0.05$).

Irradiance exposition time (h)	F_v/F_m		
	Control	500 µM Mn	1500 µM Mn
0	0.815 ± 0.003 ^{A}	0.812 ± 0.004 ^{A}	0.780 ± 0.014 ^{A}
1	0.761 ± 0.012 ^{A}	0.750 ± 0.002 ^{A}	0.677 ± 0.014 ^{B}
2	0.752 ± 0.007 ^{A}	0.731 ± 0.002 ^{A}	0.619 ± 0.028 ^{B}
4	0.685 ± 0.036 ^{A}	0.689 ± 0.004 ^{A}	0.388 ± 0.042 ^{C}

Table 5. Effects of Mn treatments on far-red light induced steady state oxidation of P700 ($\Delta A_{820\text{-}860}$, $P700^+$), the relative intersystem electron donor pool size to PSI (e^- /P700) and half times for $P700^+$ reduction ($t_{1/2}$)) of *Arabidopsis* leaves at 21 days of treatment. Different lower-case letters indicate significative differences between the Mn treatments ($P< 0.05$).

Mn Treatments (µM)	$\Delta A_{820\text{-}A860}$ ($P700^+$)	e^- /P700 (MT_{AREA}/ST_{AREA})	$t_{1/2}$ (s)
Control	423.2±7.9 a	20.7± 0.9 a	0.670 ± 0.05 b
50	416.4±8.3 a	15.7 ± 0.6 b	0.812 ± 0.06 ab
500	368.4±8.3 b	16.4 ± 0.6 b	0.938 ± 0.08 ab
1000	293.4±8.4 c	15.6 ± 0.5 b	0.908 ± 0.07 ab
1500	292.9±4.2 c	13.0 ± 0.3 c	0.984 ± 0.08 a

Figure legends:

Figure 1. Total biomass (leaves and roots, g DW) of *Arabidopsis thaliana* plants subjected to Mn treatments (μM) at the end of experiment (21 days). Different lower-case letters indicate significative differences among the Mn treatments ($P \leq 0.05$).

Figure 2. Mn concentration in leaves and roots of *Arabidopsis thaliana* plants subjected to Mn treatments (μM) at the end of experiment (21 days). Different lower-case letters indicate statistically significant differences among the Mn treatments ($P \leq 0.05$).

Figure 3. Thermoluminescence (TL) glow curves of of $S_2/S_3Q_B^-$ charge recombinations in control (solid lines) and Mn-treated (500 μM Mn^{2+} - dashed lines, 1500 μM Mn^{2+} - dotted lines) *Arabidopsis* leaves after illumination with two single turnover flashes. TL glow curves were recorded immediately after illumination. The presented glow curves are averages from 4 independent measurements.

Figure 4. Typical traces of *in vivo* measurements of P700 oxidation by far-red light (FR) in control (A, 18 μM Mn) and Mn-treated (B, 1500 μM Mn) *Arabidopsis* plants. After reaching a steady state level of $P700^+$ by FR light, single turnover (ST) and multiple turnover (MT) pulses of white light were applied. Arrows indicate application of ST, MT, FR light sources. The measurements were performed at the growth temperature of 20°C.

Figure 5. A - Representative immunoblots of SDS-PAGE separated proteins from thylakoid membranes probed with antibodies raised against PSI (PsaA and PsaB), PSII (D1, PsbO, Lhcb1)-associated polypeptides and Rubisco (PbcL) in control (C, 18 μM Mn) and *Arabidopsis* plants treated with 500 and 1500 μM Mn. B - The relative polypeptide abundance was quantified by densitometric analysis (area x intensity bands) of the immunoblots and the presented data were normalized to the relative abundance of PsaA, PsaB, D1, PsbO, Lhcb1 and PbcL in control *Arabidopsis* plants. Mean values ± SE were calculated from 3 independent experiments. Different lower-case letters indicate significative differences between the Mn treatments ($P < 0.05$).

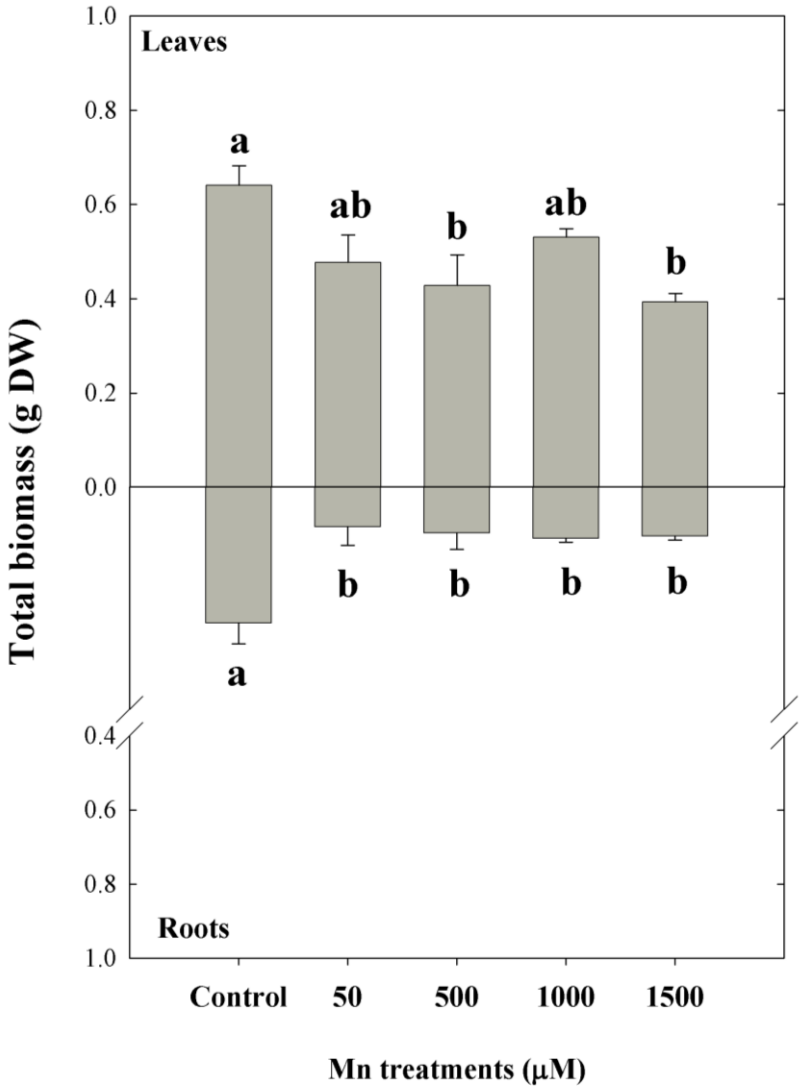
Leaves
Roots
Total biomass (g DW)
1.0
0.8
0.6
0.4
0.2
0.0
0.4
0.6
0.8
1.0
a
ab
b
ab
b
b
b
b
b
a
Control
50
500
1000
1500
Mn treatments (μM)

Figure 1

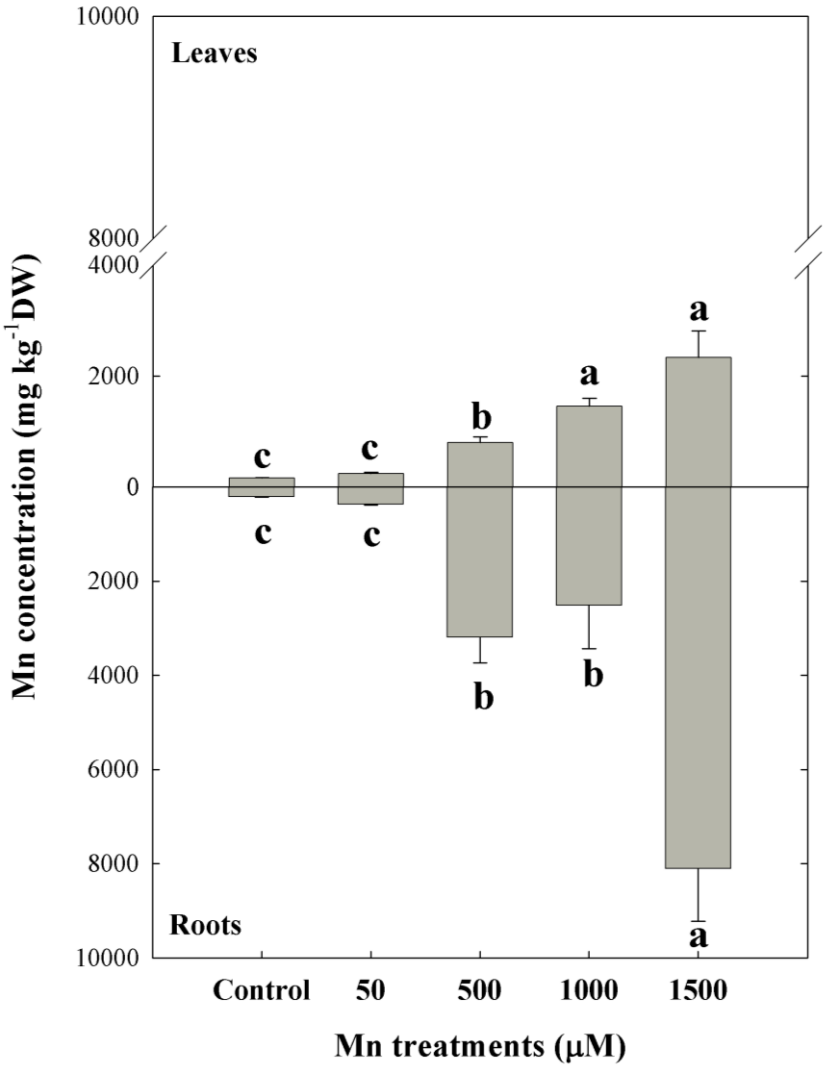
Leaves
Roots
Mn concentration (mg kg-1DW)
10000
8000
4000
2000
0
2000
4000
6000
8000
10000
a
a
b
c
c
c
c
b
b
a
Control
50
500
1000
1500
Mn treatments (μM)

Figure 2

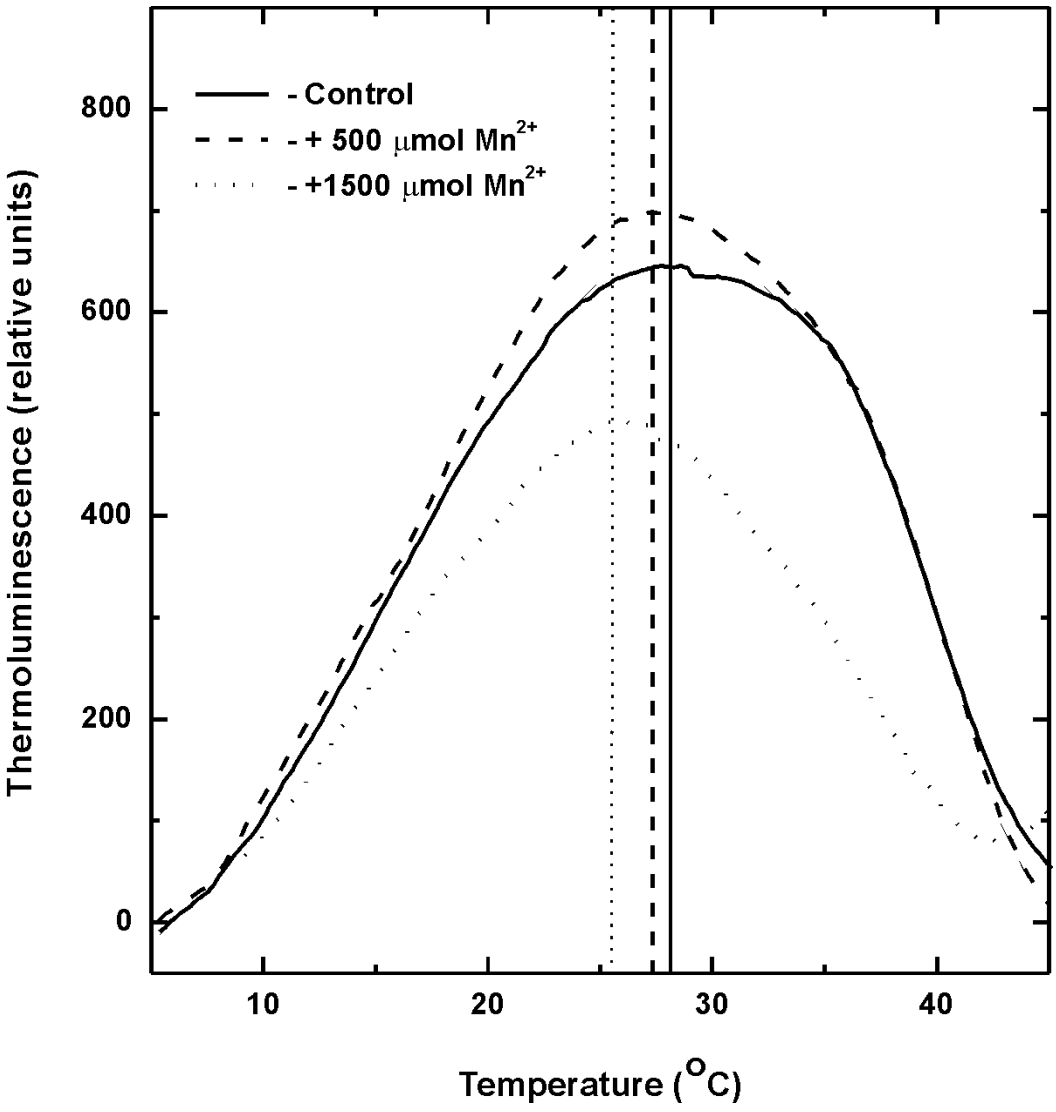
- Control
- + 500 μmol Mn2+
- +1500 μmol Mn2+
Thermoluminescence (relative units)
800
600
400
200
0
10
20
30
40
Temperature (°C)

Figure 3

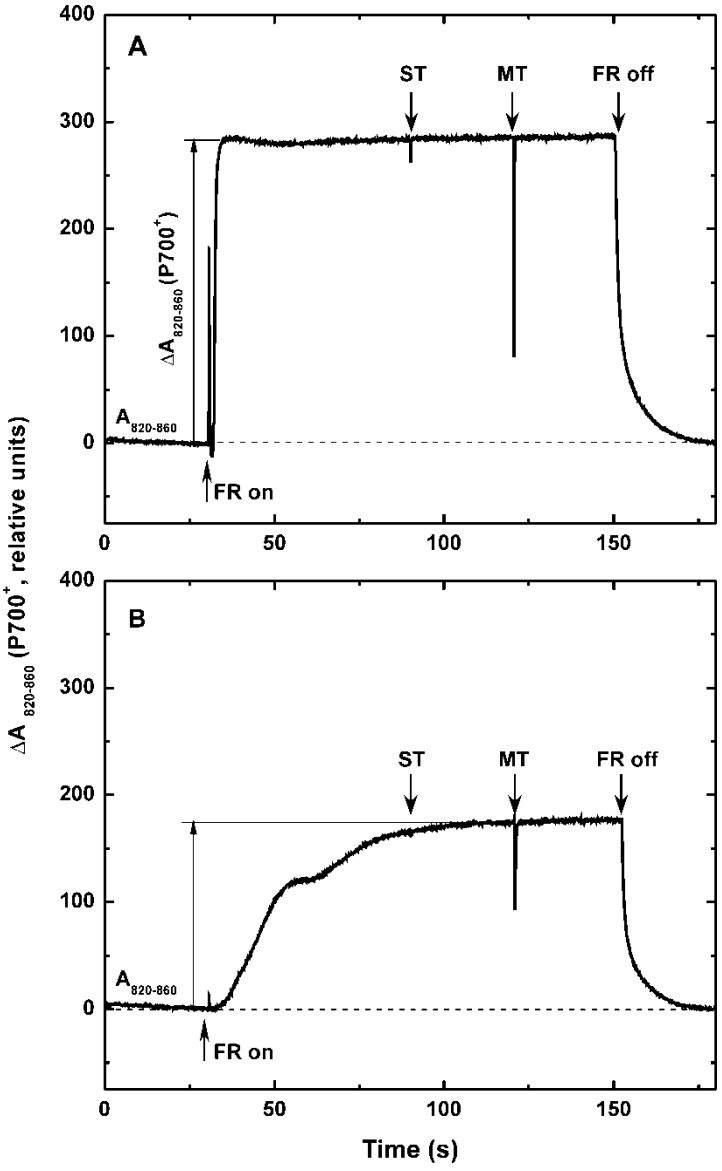
A
B
ST
MT
FR off
FR on
$A_{820-860}$
$\Delta A_{820-860}$ ($P700^+$)
$\Delta A_{820-860}$ ($P700^+$, relative units)
Time (s)
0
50
100
150
200
300
400

Figure 4

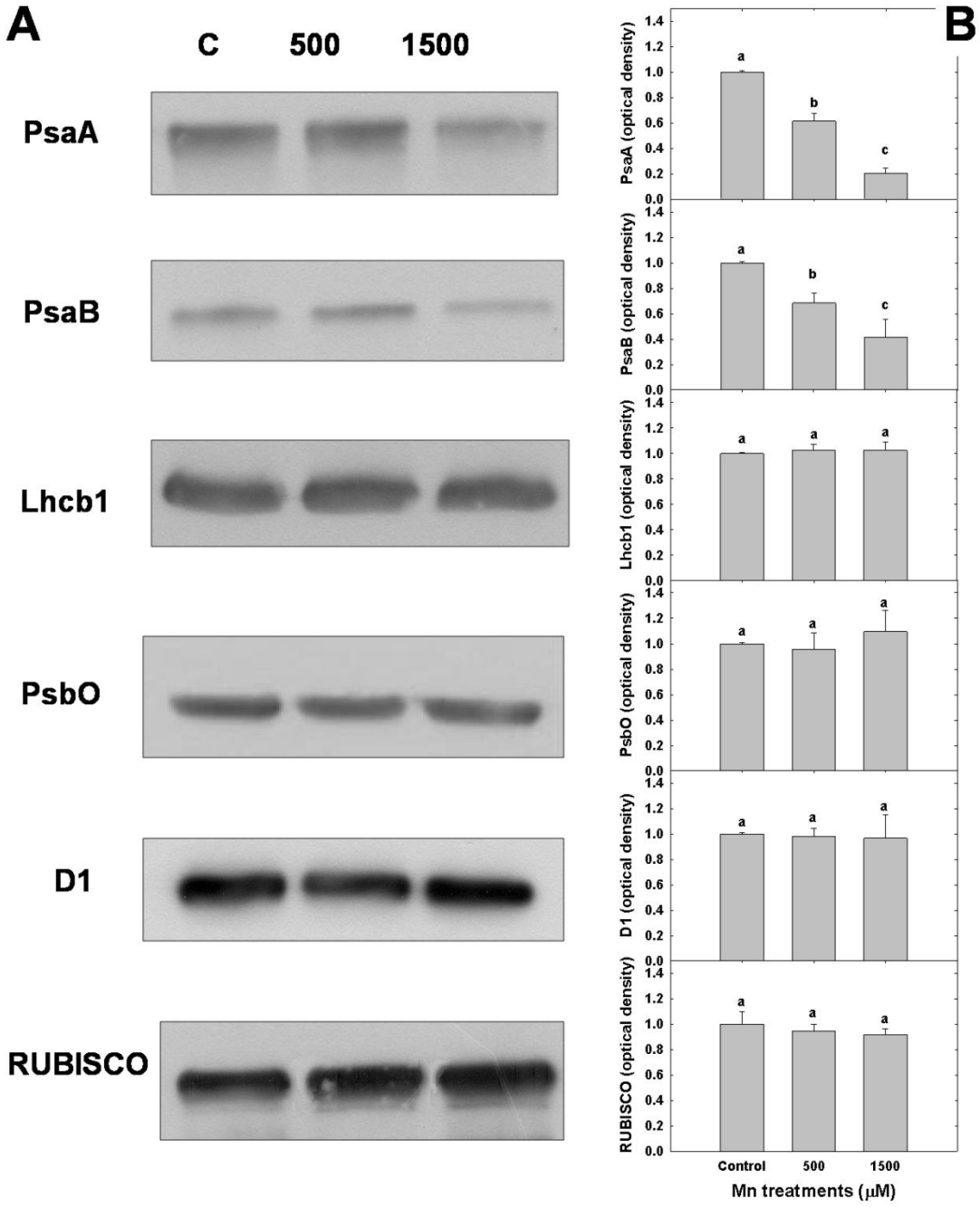
A
C
500
1500
PsaA
PsaB
Lhcb1
PsbO
D1
RUBISCO
B
PsaA (optical density)
PsaB (optical density)
Lhcb1 (optical density)
PsbO (optical density)
D1 (optical density)
RUBISCO (optical density)
Control
500
1500
Mn treatments (μM)

Figure 5.

Chapter 5:

General discussion

5.1 General discussion

In this thesis we analyzed biochemical and physiological responses of plants subjected to increasing Mn treatments growing in nutrient solution. In Chapter 3, three cultivars of highbush blueberry (*Vaccinium corymbosum* L.): 'Legacy', 'Brigitta' and 'Bluegold' were stablished under greenhouse conditions in nutrient solution with increasing Mn treatments: 2 µM (Control), 10, 50, 250, 500 and 1000 µM Mn. The pH used was 4.8. Results showed that shoots dry weight (DW) of Legacy and Brigitta were unchanged, whereas Bluegold had a significant and gradual reduction (~50%) of shoot DW with increasing Mn treatments (See Model; Fig. 5.1). Decreases in DW due to Mn excess are also observed in other plant species as rice (Horiguchi, 1988), ryegrass (Mora et al., 2009), lucerne (Gherardi and Rengel, 2004), pummel (Li et al., 2010), *Cucumis* species (Shi et al., 2005; 2006), maize (Stoyanova et al., 2009) and barley (Alam et al., 2006). According Rosas et al. (2007, 2011), detrimental effects on biomass are observed in prairie species subjected to Mn excess with stronger decrease in shoots than in roots, with visual symptoms on leaves. Similarly in maize, the DW of Mn-sensitive maize cultivar was affected mainly in shoots than roots.This DW reduction is a tipical injury caused by Mn excess. Thus, this symptom is different to other metals behavior (i.e. copper and aluminum), which tissues DW are reduced significantly at root level (Doncheva et al., 2009). In our study, Legacy showed a high Mn content in roots compared with other cultivars, suggesting a major tendency to accumulate Mn in roots (See model; Fig. 5.1). Similarly with our results, Mora et al. (2009) observed that tolerant ryegrass accumulated higher Mn concentrations in roots than in shoots. These results suggest an internal mechanism of control on Mn concentration and a lower Mn translocation to shoots in Mn-tolerant than Mn-sensitive ryegrass cultivar, which had a great translocation to shoot. In other Mn-tolerant plant species, also is relatively low the Mn translocation to aerial parts (Li et al., 2010). Thus, it can be considered that Legacy is a Mn-tolerant genotype with a low translocation of Mn to shoots, whereas Bluegold is Mn-sensitive genotype, with a major Mn translocation (See Model; Fig. 5.1).

The fluorescence parameters of the PSII (Fv/Fm, ΦPSII) showed a decrease in leaves of blueberry cultivars with the increasing Mn doses mainly in Bluegold and Brigitta (Table 1). Researches made with other metal, suggest a photoinhibition by loss the PSII efficiency, when a decrease in Fv/Fm was observed in plants subjected to cadmium (Cd) (Pagliano et

al., 2006). Excess Mn decreased significantly ΦPSII in cucumber seedlings, suggesting a decline of carbon metabolism capacity and/or a low consumption of ATP and NADPH in Calvin cycle (Feng et al., 2009; Kitao et al., 1997). Doncheva et al. (2009) found a decrease in Fv/Fm and an increase in NPQ values in the Mn-sensitive cultivar of maize, suggesting a photoinhibition by Mn excess. On the other hand, net photosynthesis and stomatal conductance of blueberry cultivars were negatively affected by increasing Mn doses in Bluegold, as was demonstrate in Mn-sensitive maize cultivars (Stoyanova et al., 2009) and cucumber plants (Feng et al., 2009). Nonetheless, early experiments in tobacco young leaves grown under Mn excess showed a decrease in net photosynthesis (Nable et al., 1988), but not associated with decreased leaf CO_2 conductance. These authors concluded that photosynthesis inhibition is an early indicator of Mn accumulation in tobacco leaves (Nable et al., 1988). With those previous researches, it could be considered to Bluegold as Mn-sensitive genotype, suggesting a major translocation to aerial part (See model, Fig. 5.1).

Abiotic stress, including Mn excess, can produce oxidative damage on plant cells leading to oxidative stress (González et al., 1998; Demirevska-Kepova et al., 2004). In our results, Legacy showed a major lipid peroxidation compared with the other cultivars; however, all the lipid peroxidation levels remained constant from the beginning until the end of Mn experiment. Different results have been reported in cucumber and wetland plants subjected to Mn excess where Mn doses increased lipid peroxidation in leaves (Shi et al., 2005; Shi and Zu, 2008; Najeeb et al., 2009). In ryegrass studies with Mn excess, lipid peroxidation was lower in Mn-tolerant ryegrass cultivars than the Mn-sensitive ones, indicating that when Mn-sensitive cutivars incremented shoot Mn concentration, a proportional augment in lipid peroxidation were observed (Mora et al., 2009). However, to prevent and reduce damage effects induced by ROS, plant has developed antioxidant defense mechanisms, which include enzymatic and non-enzymatic systems (Foyer and Noctor, 2003; Apel and Hirt, 2004). In the present study, radical scavenging activity (RSA) in blueberry cultivars with Mn excess had differences in leaves and roots, being higher in leaves than in roots in all cultivars. Interestingly, Legacy had an increase of RSA, even though TBARS amount remained unchanged, while in Bluegold the RSA was proportional to its TBARS values, reaching maximum at the highest Mn treatment (See model; Fig. 5.1). It is noteworthy that

the same cultivars used in this work and subjected to Al toxicity showed that Bluegold is the Al-sensitive cultivar, with the greatest values of TBARS without changes in RSA (Reyes-Diaz et al., 2010). The authors suggest that Legacy is Al-tolerant cultivar due to the possibility to activate defense mechanism, which contribute to decrease lipid peroxidation. Specifically, a decrease in enzyme activities as SOD was observed in Bluegold subjected to Mn excess.

Organic acid exudation in plants is plant-species specific and depends on the magnitude of stress (Jones, 1998). It has different functions as the uptake of some nutrients as P (Hoffland, 1992; Johnson et al. 1996) and the metal detoxification, where the responses dependend highly of the stress- and plant-species (Jones, 1998). Organic acids associated to Mn detoxification are oxalate, citrate, malate and succinate (Horst et al. 1999). In our study, four organic acid exudates were analyzed. From these, oxalate presented a mayor amount of exudates in all cultivars. Mora et al. (2009) found that Mn-tolerant ryegrass had the highest oxalate exudation rate at increasing Mn excess. Those responses suggest that oxalate can present an internal Mn-tolerance mechanism. High citrate exudation was found also in Mn-tolerant ryegrass, associated to the high affinity of citrate for Mn^{2+} in the rizosphere. In general, researches indicate that these organic anions exudation are released under heavy metals toxicity (lead and Cu), protecting the roots by chelating cations forming nontoxic complexes and enhanced the tolerance (Ryan et al. 2001).

For a better understanding of the results obtained in this chapter, we have designed a model that summarizes the main mechanisms involved in Mn toxicity in highbush blueberry (Fig. 5.1). In this model it is observed that Mn available is more abundant in roots of the Mn-tolerant cultivar than the sensitive ones, appearing a lower translocation from roots to leaves in Mn-tolerant cultivar. Probably, the excess of Mn^{2+} in roots, are inactivated by organic acid anions via chelation by mainly oxalate in the Mn-tolerant cultivar. In Mn-sensitive cultivar, exudation of organic acid anions did not change. With respect to SOD activity in leaves, it was no affected in Mn tolerant cultivar; whereas, in the Mn-sensitive cultivar there is a tendency to strongly decrease of the SOD activity when compared with the control at the highest Mn treatment.

In Chapter 4, we analize how a Mn excess inhibits differentially the photosystems in *Arabidopsis thaliana.* Interestingly, while reduced CO_2 assimilation induced by Mn excess has been reported in many species, it is considered one of the major physiological effects of Mn toxicity on plants (Nable et al., 1988; González and Lynch, 1997; Kitao et al., l997*a*; Lidon et al., 2004; Macfie and Taylor, 1992; Li et al., 2010). The functional integrity of the photosynthetic apparatus assessed by the maximal efficiency of PSII photochemistry (F_v/F_m) did not decline as a result of exposure to excess Mn in several plant species (Nable et al., 1988; Kitao et al., 1997b; Subrahmanyam and Rathore, 2000; Hajiboland and Hasani, 2007; Doncheva et al., 2009). However, some studies have reported a substantial decrease in F_v/F_m as a result of excess of Mn treatment in *Citrus* species (Papadakis et al., 2007; Li et al., 2010), rice (Lidon et al., 2004), Mn-sensitive maize (Doncheva et al., 2009) and cucumber (Feng et al., 2009). These results clearly indicate that the excess Mn- induced decline in CO_2 assimilation may or may not be accompanied by changes in PSII photochemistry and that response is species dependent. The experimental data presented in our study also failed to demonstrate any substantial effect of excess Mn within a wide range of Mn concentrations on the maximum efficiency of PSII photochemistry in *Arabidopsis* plants. Assessing the relative abundance of PSII-associated proteins also showed no changes in the immuno-detectable amounts of PSII-reaction center protein D1, the light harvesting chlorophyll-protein complex of PSII (Lhcb1) and the manganese stabilizing 33kDa-protein of the water splitting complex of PSII (PsbO) polypeptides in plants exposed to high Mn concentrations compared to control. In addition to the lack of significant inhibitory effects of excess Mn on PSII photochemistry, an earlier study reported that the photochemistry of photosystem I (PSI) and the photosynthetic electron transport were not significantly affected during early development of Mn toxicity in tobacco plants (Nable et al., 1988). However, a decreased Hill activity in isolated chloroplasts was found in mungbean leaves exposed to toxic Mn concentrations (Sinha et al., 2002). More recently, Li et al. (2010) have suggested that Mn excess can effectively impair the whole photosynthetic electron transport chain, thus restricting the production of reducing equivalents and limiting the rate of CO_2 assimilation in *Citrus grandis* seedlings.

Despite these few reports, the potential effect(s) of excess Mn on the functional/structural integrity of PSI remains elusive. To our knowledge, the results presented in our study are the first report of an *in vivo* assessment of high Mn concentrations on PSI photochemistry. In contrast to PSII photochemistry, *in vivo* measurements of the oxidation state of P700 ($P700^+$) (Klughammer and Schreiber, 1991; Ivanov et al., 1998), the primary donor of PSI demonstrated that the relative amount of oxidizable P700 ($P700^+$) decreased by 30% in Mn-treated *Arabidopsis* plants at concentrations above 1000 μM. The functional impairment of PSI photochemistry by excess Mn was accompanied by a significant reduction in the abundance of PSI reaction center polypeptides (PsaA and PsaB). This clearly indicates that the major target of Mn toxicity within the photosynthetic electron transport chain of *Arabidopsis* is PSI rather than PSII related components. The reduced amounts of PSI reaction center polypeptides PsaA and PsaB would imply acceptor side limitations of the photosynthetic electron transport and this could explain the increased reduction state Q_A in Mn-stressed plants reported earlier (Kitao et al., 1997b).

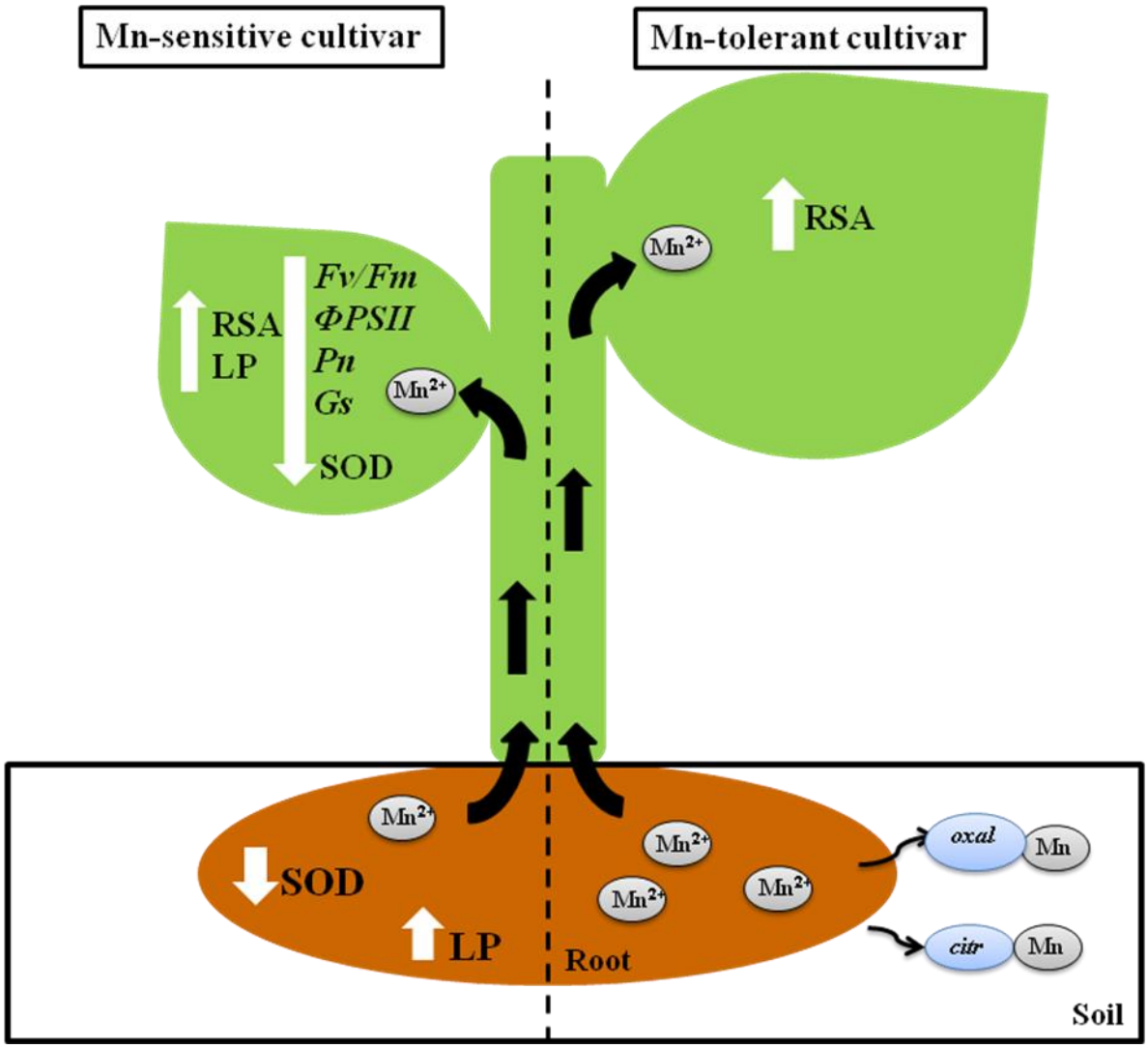

Figure 5.1. Theoretical model showing effect of Mn excess on physiological and biochemical features in leaves and roots of Mn- tolerant and Mn-sensitive blueberry cultivars. The left side of the model represents the behaviour in leaves and roots of the Mn-sensitive blueberry cultivar, whereas in the right side the changes of Mn-tolerant blueberry cultivar in leaves and roots are shown. Note that the size of leaves of the tolerant cultivar is greater than the sensitive ones. Black arrows represent the possible direction of transport Mn ions, (grey circles) into the plants. White arrows showed an enhancement or decrease of specific parameters. Blue circles indicate organic acid anions exudates in roots (*oxal*: oxalate; *citr*: citrate). Other abbreviations of parameters are: Fv/Fm: maximal photochemical efficiency of PSII; ΦPSII: effective photochemical efficiency of PSII; *Pn*: net photosynthesis; g_s: stomatal conductance; SOD: superoxide dismutase enzyme activity;

RSA: radical scavenging activity; LP: lipid peroxidation. In the Mn tolerant cultivar, parameters which did not changed or with a little variation were not represented in the model. It is important to note for a better comprehensive that the present model was performed having into account the comparison between the control and Mn treated plants with the higher or the highest Mn treatment(s).

Chapter 6:

General conclusions

6.1 General conclusions

According to main results in this thesis, it is concluded that:

- Mn excess affects differently the physiological and biochemical features of blueberry cultivars. Legacy was the most Mn-tolerant cultivar compared with Brigitta and Bluegold, being the last cultivar the most Mn-sensitive. This assumption is based on the major Mn accumulation in Legacy roots, maintaining its growth and photosynthesis. Likewise, this cultivar showed an enhancement of antioxidant capacity, maintaining lipid peroxidation at increasing Mn treatments. In addition, oxalate and citrate were the most important organic acid anions exudated, incrementing its concentration gradually with the augment of Mn doses in Legacy.
- Additionally, results of Mn excess on photosynthetic performance in *A. thaliana* indicated that the exposure of *Arabidopsis* plants to excess Mn causes specific negative effects on the abundance of polypeptides comprising the reaction center of PSI, thus resulting in decreased PSI photochemistry and lower capacity for PSI-dependent cyclic electron transport, which may be due to a Mn-induced Fe-deficiency and may have critical physiological implications under conditions of Mn toxicity in higher plants.

6.2 Futures perspectives

The knowledge presented here will contribute to understand the mechanism of Mn resistant in plants, which is very important because this element is an essential micronutrient and on the other hand, it is toxic when, is in excess depending on the acidity condition of the soil. Likewise, the understanding of this mechanism will also contribute to improving the yield and quality of cultivated plants in acid soils. Future efforts for developing Mn-tolerant plants should take all the physiological and biochemical processes into account, helping the farmers to improve the nutrition quality of plants.

6.3 General references

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APPENDIX

MANGANESE AS ESSENTIAL AND TOXIC ELEMENT FOR PLANTS: TRANSPORT, ACCUMULATION AND RESISTANCE MECHANISMS

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ABSTRACT

Manganese is an essential element for plants, intervening in several metabolic processes, mainly in photosynthesis and as an enzyme antioxidant-cofactor. Nevertheless, an excess of this micronutrient is toxic for plants. Mn phytotoxicity is manifested in a reduction of biomass and photosynthesis, and biochemical disorders such as oxidative stress. Some studies on Mn toxicity and Mn translocation from soil to plant cells in Mn^{2+} form have demonstrated their importance under low pH and redox potential conditions in the soil. When Mn is inside the cells, mechanisms that can tolerate this toxicity are also observed, being important the compartmentalization of this metal in different organelles of shoot and leaf plant cells. A key role of antioxidative systems in plants in relation to high Mn amounts has also been reported as a defense mechanism. The purpose of this review is to show the role of Mn as an essential micronutrient and as a toxic element to higher plants as well as to their transport and tolerance mechanisms. The forms and dynamics of this element in soils and the importance of the acidity for this dynamic and availability for plants are also given.

Keywords: Manganese, Mn toxicity, resistance mechanisms

INTRODUCTION

Manganese (Mn) is an essential micronutrient in most organisms. In plants, it participates in the structure of photosynthetic proteins and enzymes. Its deficit is dangerous for chloroplasts because it affects the water-splitting system of photosystem II (PSII), which provides the necessary electrons for photosynthesis (Buchanan, 2000). However, its excess seems also to be particularly damaging to the photosynthetic apparatus (Mukhopadhyay and Sharma, 1991). Thus, Mn has two roles in the plant metabolic processes: as an essential micronutrient and as a toxic element when it is in excess (Kochian *et al*., 2004; Ducic and Polle, 2005). Mn toxicity is favored in acid soils (Pendias and Pendias, 1992). With decreasing pH, the amount of exchangeable manganese – mainly Mn^{2+} form – increases in the soil solution. This Mn form is available for

plants and can be readily transported into the root cells and translocated to the shoots, where it is finally accumulated (Marschner, 1995). In contrast, other forms of Mn predominate at higher pH values, such as Mn (III) and Mn (IV), which are not available and cannot be accumulated in plants (Rengel, 2000).

Excessive Mn concentrations in plant tissues can alter various processes, such as enzyme activity, absorption, translocation and utilization of other mineral elements (Ca, Mg, Fe and P), causing oxidative stress (Ducic and Polle, 2005; Lei *et al*., 2007). The threshold of Mn injury as well as the tolerance to an excess of this metal is highly dependent on the plant species and cultivars or genotypes within a species (Foy *et al*., 1988, Horst, 1988).

The purpose of this review is to illustrate the most current understanding about Mn role as an essential micronutrient and as a toxic element to higher plants, the long distance and cellular transport in plants as well as the mechanisms or strategies involved for to resist an overload of this metal. The forms and dynamics of this element in soils and the importance of the acidity for this dynamic and availability to plants are also given.

MANGANESE FORMS AND DYNAMICS IN SOILS

Manganese biogeochemistry in soils is complex, because it is present in several oxidation states (0, II, III, IV, VI and VII), while in biological systems it occurs preferably as II, III and IV. Divalent manganese (Mn II) is the most soluble species of Mn in soil, whereas the solubility of Mn III and Mn IV are very low (Guest *et al*., 2002). Mn oxides can form co-precipitates with iron (Fe) oxides, exhibiting amphoteric behavior. In addition, Mn interacts both with cations and anions in oxidation-reduction reactions involving Mn. These reactions are influenced by a variety of physical, chemical and microbiological processes (Bradl, 2004).

Both pH and redox conditions influence Mn bioavailability in soils (Marschner, 1995; Porter *et al*., 2004). In most acid soils at low pH (<5.5) and an increased redox potential of Mn, oxides can be easily reduced in the soil exchange sites (Kogelmann and Sharpe, 2006), increasing the concentration of soluble Mn^{2+} (Watmough *et al*., 2007), which is the predominant Mn form in the soil solution (Adriano, 2001) and the most available Mn form for plants (Marschner, 1995). At higher soil pH (up to pH 8), chemical Mn^{2+} auto-oxidation is favored over MnO_2, Mn_2O_3, Mn_3O_4 and even Mn_2O_7, which are not normally available to plants (Ducic and Polle, 2005; Humpries *et al*., 2007; Gherardi and Rengel, 2004). Furthermore, high pH allows Mn adsorption into soil particles, decreasing their availability (Fageria *et al*., 2002). Nevertheless, some reports have suggested that an excess of available Mn is produced under reduced soil conditions, even at high soil pH values (Hue, 1988). A reducing environment can be produced when there is an excess of water, poor drainage or applications of organic material (Hue, 1988; El-Jaoual and Cox, 1998). Different organic molecules can dissolve solid Mn oxides through transfer of electrons, transforming them into an available Mn form for plants (Laha and Luthy, 1990). Soil acidification is also accentuated by abundant pluviometry during winter, causing the main cations to leak from the soil (Mora *et al*., 2006). On the other hand, lime application is a key factor in decreasing soluble Mn in acid soils with a high Mn content, given that it can increase soil pH (Hue and Mai, 2002).

The total Mn content in soils is variable. Sparks (1995) reported small amounts of Mn in soils, fluctuating from 20 to 10,000 mg kg^{-1} soil, whereas other authors have registered total Mn contents between 450 and ~ 4,000 mg Mn kg^{-1} soil (Adriano, 2001). In addition, the total Mn soil content was from 15 to 17 mg kg^{-1} in acid soils without liming (pH about 4.4) (Hue and Mai, 2002). In liming acid soils, the interchangeable Mn concentration varied from 14 to 96 mg kg^{-1} soil in one year, with higher concentrations under high moisture and temperature conditions (Conyers *et al*., 1997). In Chilean volcanic soils, so-called Andisols, Mn concentrations fluctuate between 4.5 and 80 mg kg^{-1} depending on the agronomic management. Moreover, the Mn amount is higher in pasture soils (up to 400 mg kg^{-1}) mainly in winter (Data from Laboratorio de Análisis de Suelo y Planta, Universidad de La Frontera, Temuco, Chile).

Environmental conditions also affect Mn soil contents. The highest concentrations of soluble and exchangeable Mn are found after hot, dry summers and under warm waterlogged conditions in acid soils. This is probably due to the inhibition of Mn-oxidizing organisms, thereby allowing the chemical reduction of Mn oxides in these soils (Sparrow and Uren, 1987; Conyers *et al*., 1997).

Manganese dynamics in the rhizosphere

Rhizosphere, which is the narrow zone of soil immediately surrounding the root system, is of great importance for mineral plant nutrition. In this zone, both the mobilization and immobilization of nutrients occur (Marschner, 1995). As shown in Figure 1, a mobilization of Mn^{2+} is produced by the rhizosphere acidification due to the release of H^+ or low molecular weight organic acids (LMWOA) from plants (Rengel and Marschner, 2005). Organic acids released in anion forms from roots can chelate Mn^{2+} released from the MnOx (Mn oxides) (Ryan *et al.,* 2001). Neumann and Römheld (2001) reported that mobilization of micronutrients (including Mn) into the rhizosphere is due mainly by its acidification and complexation with the organic acids (citrate) in various plant species. It has been reported that organic amendments (chip compost and pine bark) applied to melon plants released organic compounds such as arabinose and malic acid that can dissolve MnO_x (Tsuji *et al*., 2006). Soil microorganisms can also help Mn mobilization and immobilization, depending on soil conditions (Marschner, 1995). In aerated soils, microorganisms may mobilize Mn through MnOx reduction favored by H^+ root excretion. In contrast, Mn-oxidizing bacteria can decrease Mn availability in aerated and calcareous soils or in poorly aerated and/or submerged soils. Another key factor in the Mn dynamics in soil is organic matter (OM). Given that OM is negatively charged, it has a great Mn adsorption capacity, forming Mn complexes which decrease the amount of exchangeable Mn. However, the Mn adsorbed by OM can be exchanged by the H^+ released from the roots (Bradl, 2004).

MANGANESE TRANSPORT AND ACCUMULATION IN PLANTS

As mentioned above, reduced Mn (Mn^{2+}) form is the only available metal form for plants. It can be taken up via an active transport system in epidermal root cells and transported as divalent cation Mn^{2+} into the plants (Marschner, 1995; Gherardi and Rengel, 2003; Pittman, 2005). Manganese uptake by roots is characterized as a biphasic process. The initial and rapid uptake phase is reversible and non-metabolic, with Mn^{2+} and Ca^{2+} or

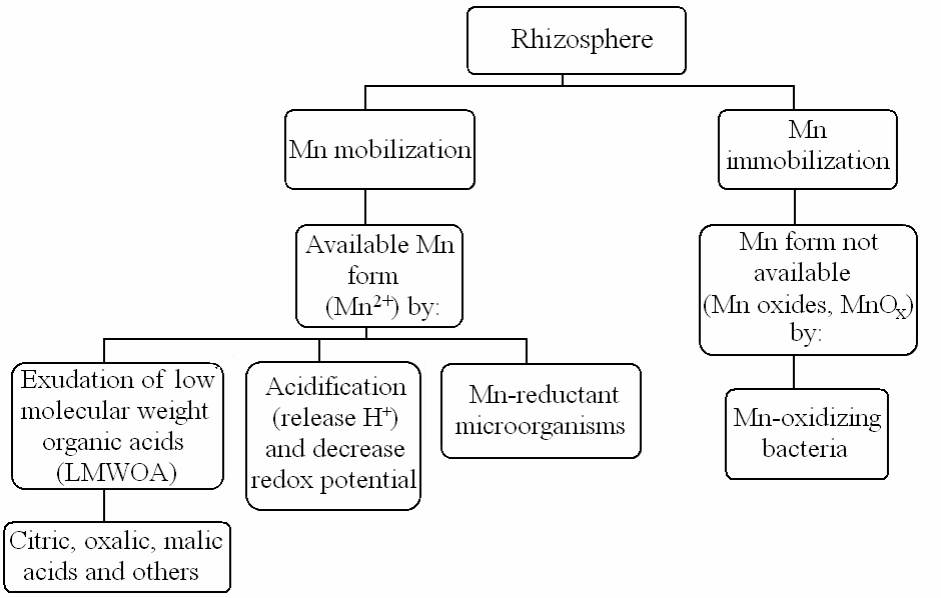

Figure 1. The role of soil rhizosphere in the mobilization and immobilization of Mn in soils.

other cations being freely exchanged in the rhizosphere. In this phase, Mn^{2+} appears to be adsorbed by the negatively charged cell wall constituents of the root-cell apoplastic spaces (Humphries *et al*., 2007; Clarkson, 1988). The second phase is slow, with Mn^{2+} being less readily exchanged. Its uptake into the symplast is dependent on plant metabolism (Maas and Moore, 1968), although the exact mechanisms are not clear (Humphries *et al*., 2007). It has been shown that in transgenic tobacco transformed with a tomato root protein with a metal binding side at its N-terminus (LeGlp1), Mn binds to this protein. This strongly suggests the involvement of LeGlp1 in Mn uptake from the soil (Takahashi and Sugiura, 2001). Kinetic measurements have demonstrated 100 to 1,000 times higher rates of Mn transport than the estimated plant requirement for this element (Clarkson, 1988). These transport rates are explained by the high capacity of ion carriers and channels in the Mn ion transportation through the plasma membrane at a speed of several hundred to several million ions per second per protein molecule (Humphries *et al.,* 2007). According to these authors, Mn distribution from root cells within the whole plant involves primary transport in the xylem, transference from the xylem to the phloem and re-translocation into the phloem. Xylem transport from roots to the above-ground parts of plants is performed by the transpiration stream, whereas phloem transport is more selective, taking place from sources to sinks (Marschner, 1995). Nonetheless, a low mobility in phloem has been reported for Mn, and its redistribution may depend on the plant species and stages of development (Herren and Feller, 1994). In fact, it has been reported that Mn transport from roots to grains is frequently insufficient at

a mature stage of wheat. The relatively poor Mn mobility in the phloem emphasizes the importance of xylem in the transport of this element, even in wheat grain discharge (Rengel, 2001).

Manganese generally tends to accumulate predominantly in the plant shoots than in the roots, as demonstrated in Mn labeling experiments with ^{54}Mn at an early stage of wheat (*Triticum aestivum* cv. Arina) development, where a fast Mn transport from roots to shoots was visualized in the xylem and was essentially immobile in the phloem (Page and Feller, 2005). Similar effects on Mn translocation have been shown by the same technique in young (28 days) white lupine plants (*Lupinus albus*) (Page *et al.,* 2006). Nevertheless, Mn was present in a large amount in the root system, hypocotyls and stem in older lupine plants, immediately after the labeling phase (day 0). Seven days later (day 7) almost all ^{54}Mn had moved to the youngest fully expanded leaves and only a small fraction to the other leaves. Mn accumulation was observed in the periphery of the oldest leaves. These authors reported that Mn was rapidly released from the roots into the xylem, reaching photosynthetically active leaves via the transpiration stream. Furthermore, the low mobility of this element via phloem in the shoot may be due to a restricted loading of soluble Mn into the phloem or by insolubilization in the leaves, although the issue remains to be clarified (Page *et al.,* 2006). Page and Feller (2005) emphasized that little is known about the mechanisms involved in the loading of Mn into the phloem and the chemical transport forms.

In addition to the long distance transport of Mn, short distance transport mechanisms are important for the translocation of this metal into the cell and cell organelles. These mechanisms involve Mn translocation throughout the plasma membrane and the biomembranes of organelles (Ducic and Polle, 2005; Pittman, 2005). Possible mechanisms of homeostasis and Mn transport, based on studies performed in yeast (*Saccharomyces cerevisiae*) cells and in *Arabidopsis thaliana* plants, have been discussed (Delhaize *et al.,* 2007; Reddi *et al*., 2009). They pointed out that transport proteins play an important role for the maintenance of adequate Mn concentrations in the cytoplasm. Moreover, a variety of metal transporter family proteins with a broad-specificity such as Fe^{2+} and Ca^{2+} transporters have also the ability to transport Mn into the plant cells (Pittman, 2005). Migocka and Klobus (2007) demonstrated the activation of an antiport system in *Cucumis sativus* with different affinities for Pb, Mn, Ni and Cd in the root plasma membrane. This antiport system participates as part of the general defense mechanism activated under heavy metal stress. Using tonoplast-enriched vesicles, Shigaki *et al*., (2003) suggested that a Cd/H antiporter might also be involved in Mn accumulation in vacuoles.

In Table 1 we have summarized some of the metal transporter proteins, their families, cellular localization and the transported metals. Transporter proteins are described according to their localization. In the plasma membrane, four Mn^{2+} uptake transporters are identified: AtIRT1, Nramp, AtYSL (Ducic and Polle, 2005; Pittman, 2005) and PHO84 (Ducic and Polle, 2005). IRT1 can transport Mn when expressed in yeast (Korshunova *et al*., 1999). Nramp is also considered a metal transporter protein that can transport Mn away from other cations. This protein may be localized in the tonoplast rather than in the plasma membranes (Thomine *et al*., 2003). AtYSL belongs to the yellow stripe-like (YSL) proteins, also involved in metal-complex transport in the plasma

membrane (Roberts *et al*., 2004). This complex can be formed by nicotianamine (NA), which is a strong chelator of metals including Mn^{2+} (Pittman, 2005). PHO84 is a transporter protein identified in *S. cerevisiae* and it has a high-affinity phosphate uptake (Mitsukawa *et al*., 1997). Luk *et al*. (2003) reported a new form in Mn transport as $MnHPO_4$. However, there is no evidence for Mn^{2+} or $MnHPO_4$ accumulation by a plant phosphate transporter (Pittman, 2005).

Some transport proteins have been related to Mn^{2+} transport and accumulation into the intracellular compartments, such as the vacuole. It has been suggested that a metal transporter (specifically antiporter CAX2, calcium exchanger 2) originally identified as a Ca^{2+} transporter (which can also transport Cd^{2+}) located in the cytosol. It has also the ability to transport Mn to the vacuole in tobacco plants (*Nicotiana tabacum*) and yeast (Hirschi *et al*., 2000; Pittman, 2005) (Table 1). ATP-binding cassette (ABC) protein transporters are considered to be involved in detoxification processes (Martinoia *et al*., 2002). Studies on cyanobacteria also suggested the putative role of these proteins in Mn^{2+} transport (Bartsevich and Pakrasi, 1996).

Another protein, considered indirectly as a metal transport protein is the ShMTP1, which is able to sequester metal ions within cells or efflux them out of the cells (Delhaize *et al*., 2003). Therefore, these authors considered it a metal-tolerant protein. Ducic and Polle, (2005) and Pittman (2005) highlighted that, despite the available information about Mn transport across membranes in plant cells, the Mn transport and efflux strategies into the mitochondria, chloroplasts and Golgi are not completely understood. Nonetheless, Mills *et al*. (2008) have recently identified a Ca-ATPase that also transports Mn^{2+} into Golgi apparatus (Table 1).

MANGANESE AS AN ESSENTIAL ELEMENT IN PLANT METABOLISM

The main Mn role in photosynthesis is its involvement in the water-splitting system of photosystem II (PSII), which provides electrons necessary for photosynthetic electron transport. In water photolysis, a group of four Mn atoms (Mn cluster) is associated with the oxygen evolving complex (OEC) bound to the reaction center protein (D1) of PSII (Goussias *et al*., 2002). The Mn cluster in PSII accumulates four positive charges, which oxidize two water molecules, releasing one O_2 molecule and four protons. Therefore, this metal cluster is considered a catalyst compound of water oxidation (Zouni *et al*., 2001), where Mn ions are close to a redox-active tyrosine residue (Z and D) (Goussias *et al*., 2002).

Manganese also plays a role in ATP synthesis (Pfeffer *et al*., 1986), in RuBP carboxylase reactions (Houtz *et al*., 1988) and the biosynthesis of fatty acids, acyl lipids and proteins (Ness and Woolhouse, 1980). In addition, Mn plays a primary role in the activation and as cofactor of various enzymes in plants (~35) (Burnell, 1988), such as: Mn-superoxide dismutase, Mn-catalase, pyruvate carboxylase and phospho-enolpyruvate carboxykinase (Ducic and Polle, 2005). Manganese is also essential for the biosynthesis of chlorophyll (through the activation of specific enzymes), aromatic amino acids (tyrosine), secondary products, like lignin and flavonoids (Lidon *et al*., 2004). It also participates in the biosynthetic pathway of isoprenoids (Lidon *et al*., 2004) and assimilation of nitrate (Ducic and Polle, 2005). Hence, Mn is involved in metabolic processes such as respiration, photosynthesis, synthesis of aminoacids and hormone activation (indol acetic acid, IAA) throughout the IAA-oxidases (Burnell, 1988).

Table 1. Some transporter proteins implicated in Mn^{2+} and other cations transport and their cellular localization. (Summarized from Ducic and Polle, 2005; Pittman, 2005 and Mills *et al*., 2008). Abbreviations as follows: At= *Arabidopsis thaliana*; Sh= *Stylosanthes hamata*).

Transporter proteins	**Protein cellular localization and protein family transporters**	**Transported ions**
AtIRT1	Plasma membrane protein (ZIP, zinc-regulated transporter/iron regulated transported (ZRT/IRT1) related protein) family transporter	Mn^{2+} and Fe^{2+}, Zn^{2+} and Cd^{2+} under Fe-deficiency conditions.
AtECA1	Endoplasmic reticulum (ER) Ca^{2+}- and Mn^{2+}-transporting P-type ATPase	Ca^{2+} and Mn^{2+}
AtCAX2	Vacuolar cation/H^+ antiporter CAX (the cation exchanger)	Mn^{2+}, Ca^{2+} and Cd^{2+}
AtNramp3	Vacuolar Nramp transporter. Also, possible plasma membrane localization (Nramp?)	Mn^{2+}, Fe^{2+} and Cd^{2+} in Fe-deficiency conditions
ShMTP1	Vacuolar-localized cation diffusion facilitator (CDF) family transporter	Related to the *Stylosanthes hamata* Mn^{2+} transporter
ABC	Vacuolar- localized ATP binding cassette transporter families	Related to the cyanobacterium *Synechocystis* Mn^{2+} transporter
AtOPT3	Probably located in the plasma membrane (AtOPT3?). It is an oligopeptide transporter-like protein (OPT).	Possible transport of Cu^{2+} and Fe^{2+} and Mn^{2+}
AtYSL	Probably located in the plasma membrane. Yellow stripe-like transporter with equivalent function to rice OsYSL2, a Mn^{2+}-nicotianamine (NA) and Fe^{2+}-NA transporter	Mn^{2+} and Fe^{2+}
AtECA3	Ca^{2+} transporters (Ca-ATPases) in Golgi.	Mn^{2+} and Ca^{2+}
PHO84	Probably located in the plasma membrane. It is $MnHPO_4$ transporter.	Mn binding to phosphate.

As a cofactor of superoxide dismutase (SOD), manganese participates in the plant's defense against oxidative stress, produced by elevated levels of activated forms of oxygen and free radicals (reactive oxygen species, ROS), which are harmful to plants. It has been proposed that Mn can act as a scavenger of superoxide (O_2^-) and hydrogen peroxide (H_2O_2). However, this mechanism is still unclear (Ducic and Polle, 2005).

Manganese SOD (MnSOD) belongs to the group of metal-containing SOD enzymes which are classified according to their metal cofactor: iron SOD (Fe-SOD), localized in the chloroplast; copper-zinc SOD (Cu/Zn-SOD), located in the chloroplast, cytosol, and possibly in the extracellular space. Manganese SOD is found mainly in mitochondria (Clemens *et al*., 2002) and peroxisomes (Alscher *et al*., 2002). MnSOD may play an important role in the adaptive responses of plant cells under environmental stresses such as salt stress, enhancing their tolerance. This tolerance has been demonstrated in transgenic plants of *Arabidopsis,* where Mn-SOD was overexpressed (Wang *et al*., 2004). Similarly, in tomato transgenic plants which overexpressed MnSOD, an improvement in tolerance to NaCl stress in seedlings was concomitant with an improvement in seed germination and root development (Wang *et al*., 2007).

MANGANESE PHYTOTOXICITY AND INJURY SYMPTOMS

As an essential micronutrient, low Mn levels are absolutely necessary for normal nutrition and development of plants. Normal Mn contents of leaves differ greatly between species (30-500 mg kg^{-1} Mn dry mass, Clarkson, 1988). Nonetheless, when it is present in excessive amounts, it is extremely toxic to plant cells (Migocka and Klobus, 2007). The injury extent of Mn toxicity is approximately proportionate to the concentration of accumulated Mn excess. However, there is considerable inter- and intra- specific variation among Mn levels that induce toxicity as well as the symptoms of this toxicity in plant species (Foy *et al*., 1988).

In addition to a decrease in growth rate, symptoms of Mn toxicity such as chlorosis in leaves (intervenial and marginal) and necrotic leaf spots are very common and have been reported in the whole plants of canola (Moroni *et al*., 2003), clover (Rosas *et al*., 2007), ryegrass (Mora *et al*., 2009) as well as in leaves of barley and cowpea (Demirevska-Kepova *et al*., 2004; Führs *et al*., 2008) (Table 2). Necrotic brown spots and chlorotic leaves are frequently reliable indicators of the severity of Mn toxicity in plants (Wissemeier and Horst, 1991). The intervenial chlorosis due to Mn toxicity can have an appearance similar to that observed under Fe deficiency (Sarkar *et al*., 2004). Moreover, the Mn toxicity is intensified when other available elements such as Ca, Mg, K, Fe and Si are in a low quantity (Abou *et al*., 2002). However, a decrease in productivity by Mn toxicity without the appearance of leaf visual symptoms is sometimes observed (Miner and Sims, 1983). It is important to know that all these symptoms induced by Mn toxicity are preceded by an alteration of the photosynthetic apparatus and the photosynthetic performance of plants.

Other studies have shown that in rice (*Oryza sativa* cv. Safari) exposed to Mn excess in a nutrient solution, Mn was predominantly accumulated in leaves compared with roots (Lidon, 2001), whereas in *Sinapis alba* Mn mostly accumulated in the shoots (Farasova and Beinrohr, 1998).

Table 2. Symptoms of Mn toxicity and Mn concentrations in organs of some plant species subjected to toxic Mn concentrations according references from the last decade (earlier references in the text). Mn treatments were performed in nutrient solutions with $MnCl_2$ or $MnSO_4$.

Species	Mn treatment	Mn concentrations in different plant organs	Symptoms of Mn toxicity	References
Rice (*Oryza sativa* L.)	583 µM	Shoots: 2020 µg g^{-1} dw	Decrease of shoot growth rate.	Lidon and Texeira (2000a)
Barley (*Hordeum vulgare* L.)	1830 µM 18300 µM	Leaves: 656 mg g^{-1} dw Leaves:1615 mg g^{-1} dw	Dark-brown necrotic spots, individually or in groups.	Demirevska-Kepova *et al.* (2004)
Ryegrass (*Lolium perenne* L.)	355 µM	Shoot: 2357 mg kg^{-1} Roots: 2408 mg kg^{-1}	Chlorotic leaves. Decrease of dry weight in roots.	Rosas *et al.* (2007)
Ryegrass (*L.* perenne L.)	150 µM	Shoot: 902 mg kg^{-1} Roots: 1342 mg kg^{-1}	Dry weight reduction. Dry weight reduction.	Mora *et al*. (2009)
Clover (*Trifolium repens* L.)	355 µM	Shoot: 2050 mg kg^{-1} Root: 7481 mg kg^{-1}	Reddish borders on leaves. Decrease of dry weight.	Rosas *et al*. (2007)
Soybean (*Glycine max* L.)	200 µM	Leaves: 806 mg kg^{-1} Roots: 502 mg kg^{-1}	Chlorotic leaves. No visual symptoms, increase in root diameter.	Lavres Jr *et al.* (2009)
Cowpea (*Vigna unguiculata* L.)	50 µM	Leaves: ~ 25 µmol g^{-1} dw	Brown spots on leaves.	Führs *et al.* (2008, 2009)
Canola (*Brassica napus* L.)	200 µM	Shoot: ~ 3500 µg g^{-1} dw	Necrotic leaf spots, chlorosis in leaf margin.	Moroni *et al*. (2003)
Juncus effuses L. (wetland plant)	500 µM	176 mg kg^{-1}	Reduction in plant dry biomass and height, no phytotoxic visual symptoms.	Najeeb *et al*. (2009)
Populus cathayana	1000 µM	Leaves: 713 mg kg^{-1} dw	Decrease in shoot height, total biomass, and total leaf area.	Lei *et al.* (2007)

Despite the importance of Mn excess in the photosynthetic performance of plants, only a few studies about this issue are available. A reduction in photosynthesis, in chlorophyll *a* and *b* contents and their biosynthesis, as well as a reduction in carotenoids is frequently found in plants and also in algae under Mn excess (Macfie and Taylor, 1992; Hauck *et al*., 2003). In rice cultivated at different Mn concentrations (from 2.3 to 583 µM), a significant decrease in chlorophyll *a* content has been reported at the highest Mn concentration (Lidon and Teixeira, 2000a). Nable *et al*. (1988) reported an early inhibition of photosynthesis concomitant with a high Mn accumulation in the leaves in *Nicotiana tabacum* cultivated in nutrient solutions with a Mn excess (1,000 µM). The authors concluded that the inhibition of photosynthesis is an early indicator for Mn toxicity in tobacco leaves. Lidon *et al*., (2004) also observed a decline in net photosynthesis (µmol CO_2 $m^{-2}s^{-1}$) and photosynthetic capacity (µmol O_2 $m^{-2}s^{-1}$) in rice plants subjected to 9.1 and 36.4 µM Mn treatment, respectively, with no changes in the levels of the ratio between variable (Fv) and maximum (Fm) chlorophyll fluorescence (Fv/Fm). However, an increase in the photochemical quenching and the quantum yield of non-cyclic electron transport was found up to 36.4 µM Mn. Since Mn is accumulated in thylakoids, this element may interfere with thylakoid stacking, decreasing net photosynthesis (Lidon and Teixeira, 2000a).

Similarly to Mn toxicity, Mn deficiency also depressed leaf photosynthetic capacity in plants of *Carya illinoinensis*, primarily by reducing the number of PSII units per unit leaf area, but these PSII units maintained abilities similar to those of the control plants (Henriques, 2003). Increased Mn concentrations (0 – 1,000 and 10,000 µM Mn) in wheat plants inhibited the biosynthesis of chlorophyll and carotenoids, inducing a decrease in photosynthetic electron transport rates and therefore a decrease in the rate of photosynthesis (Macfie and Taylor, 1992). Higher Mn concentrations are also involved in a shortening of root and shoot and in a decreased chlorophyll concentration in the Mn accumulator plant, *Alyssum murale* (Abou *et al*., 2002). Recently, Amao and Ohashi (2008) suggested that high Mn amounts in spinach leaves inhibited the activity of oxygen evolved complex of PSII.

Manganese toxicity and oxidative stress

Mn toxicity can also trigger oxidative stress in plant cells (Demirevska-Kepova *et al*., 2004). As a toxic metal, Mn can cause metabolic alterations and macromolecular damage that disrupt the cell homeostasis (Hegedüs *et al*., 2001; Polle, 2001). According to Lynch and St. Clair (2004), Mn toxicity in plants generates reactive oxygen species (ROS), mainly $OH^{\cdot}$, the most reactive oxidant and harmful species in cells (Lidon and Henriques, 1993). With respect to the oxidative stress responses to Mn excess, Lidon and Teixeira (2000b) reported that at the first growth stages of rice, two kinetic phases can be distinguished: in the first one, there is an increase in Mn accumulation in the thylakoid lamellae (McCain and Markley, 1989), which inhibits electron leakage from the Hill and Mehler reactions, limiting ROS formation. In the second kinetic phase, higher Mn amounts inhibit the non-cyclic photophosphorylation process, promoting an increase in ROS production that parallels an injury increase in the thylakoid peroxidase system (Lidon and Teixeira, 2000b). These authors concluded that Mn excess increases the disorganization in chloroplast lamellae,

but elevated activity of superoxide dismutase (SOD) still limits cell damage.

It has also been reported that in *Cucumis sativus* plants both Mn excess and optimum light intensity determine an enhancement in oxidative stress by increased Mn content in the tissues concomitant with an inhibition of plant growth (Shi *et al*., 2006). Investigations performed by González *et al*., (1998) showed that lipid peroxidation was not induced by Mn-toxicity stress in the mature leaves of *Phaseolus vulgaris*, although other studies have shown that lipid peroxidation occurred in isolated chloroplast of wheat (Panda *et al*., 1986). However, González *et al*. (1998) mentioned that this damage process could be related to the development stage of leaves, with the damage being more intense in immature than in mature leaves.

The geographical origin of the species and climatic conditions also affect the degree of Mn toxicity in plant species or populations as shown in two populations of *Populus cathayana*, coming from a wet and dry climate cultivated in an acid solution with increasing Mn concentrations (Lei *et al*., 2007). The results showed that the wet climate population accumulated more Mn in plant tissues especially in leaves, decreasing their growth, chlorophyll contents, and activities of antioxidant enzymes than the dry climate population.

TOLERANCE MECHANISMS TO MANGANESE TOXICITY

The ability of plant for to grow and survive in a metal-contaminated environment, commonly called resistance, can be achieved through different mechanisms: avoidance and/or tolerance. The former involves a protective role that prevents the metal ions from entering the cytoplasm of plant cells (Blamey *et al*., 1986; Marschner, 1991). The latter strategy (tolerance) implies a detoxification of metal ions after they have crossed the plasma cell membrane or internal organelle biomembranes (Macfie *et al*., 1994). However, as shown in the following paragraphs, the differentiation of these mechanisms in the pertinent literature is very confusing.

The sequestering of Mn in the apoplast is considered an avoidance mechanism. However, some researchers have included this feature as a tolerance mechanism. For example, Horst *et al*., (1999) suggested that tolerance to Mn excess in *Vigna unguiculata* is performed by the reduction of Mn^{2+} activity in the apoplast throughout complexation by organic acids. In this species, symptoms such as brown leaf cell spots are also identified as oxidized Mn, and phenolic compounds present in the cell walls are considered a Mn tolerance mechanism (Wissemeier and Horst, 1992). On the other hand, the Mn^{2+} oxidation by peroxidases in the cell walls of roots is considered by Marschner (1991) as an avoidance mechanism, although the existence of such a mechanism was not considered in the study of Horiguchi (1987). In this study, it is suggested that oxidized Mn deposition in plant tissues corresponds to a tolerance mechanism to Mn toxicity, with *Cucumis sativus* being more tolerant to high Mn deposition in tissues than melon (*Cucumis melo)*. Blamey *et al*., (1986) reported the accumulation and secretion of Mn^{2+} in and around the trichomes of sunflower plants (*Helianthus annuus*) as a Mn tolerance mechanism. Another strategy that plants use to prevent the toxic effects of heavy metals as well as of Mn can be the efflux from the cell. In this process, the Mn cell is delivered into the Golgi apparatus and finally exported from the cell via secretory pathway vesicles that carry the metal to the cell surface (Ducic and Polle, 2005).

Summarizing the available literature regarding tolerance mechanisms to Mn, it appears that the main Mn tolerance mechanism is the sequestration by organic compounds in metabolically less active cells or organelles. The vacuole is considered the biggest and most important compartment, because it can store many toxic compounds (Pittman, 2005). Hence, an increase in phenolic compounds was found in the hydrophyte (*Trapa natans*) leaves exposed to high Mn levels (130 µM) (Baldisserotto *et al*., 2004). These compounds chelate Mn inside the vacuole, segregating the metal ion in the protoplasm and thus reducing the damage (Davis *et al*., 2001). A similar key role has recently been assigned to oxalic acid in Mn internal sequestration by chelating specifically the Mn excess in vacuoles of Mn hyperaccumulator plants (*Phytolacca americana*) (Dou *et al*., 2008). Furthermore, it has been observed that a Mn excess can accumulate dark material in the vacuoles, probably for deposition of Mn oxides or an increase in polyphenol oxidase activity in *Citrus volkameriana* plants (Papadakis *et al*., 2007). Similarly, studies about the effect of Mn excess in varieties of the conifer *Pseudotsuga menziesii* showed dark deposits of Mn-complexes in plant root vacuoles, which were associated with phosphate, establishing "free" Mn^{2+} to form insoluble complexes, giving a greater tolerance (Ducic and Polle, 2007). Additionally, these studies showed that both, root elongation and biomass production were inhibited by Mn treatment above 2,500 µM, mainly in *P. menziesii* var. *glauca*, confirming that it is a species with lower tolerance than *P. menziesii* var. *viridis.*

Another strategy to confer Mn tolerance inside plant cells is associated with several metal transporter proteins identified in the Mn transport mechanisms described in section 4. Metal transporter proteins located in the tonoplast (CAX2 in tobacco plants, ShMTP1 in *Arabidopsis* plants) conferred greater tolerance on elevated Mn^{2+} levels due to the internal sequestering of this element (Hirschi *et al*., 2000; Delhaize *et al*., 2003). Other transporter proteins (ECA1) can maintain low cytosolic Mn, since it moves into the endoplasmic reticulum (Wu *et al*., 2002). In this research, ECA1 was able to reduce cytosolic Mn^{2+}, preventing an interference with the internal distribution of other ions (Mg^{2+}, Fe^{2+} or Ca^{2+}).

Unlike other metal stresses, the accumulation of Mn excess does not have a single cell target. Depending on the plant species, different organelles can serve as stores for this accumulation (Lidon *et al*., 2004). Under high Mn levels apart from vacuoles, chloroplasts are important sinks of this metal in *Citrus volkmeriana*. This feature, together with the larger size of this organelle, is considered to be an adaptive response of this plant to Mn excess (Papadakis *et al*., 2007). In general, cultivated plants like rice are considered tolerant to Mn toxicity (Lidon, 2001) because their leaf tissues can accumulate from 5 to 10 times more Mn than other grasses (Foy *et al*., 1978). The Mn tolerance mechanism in this species included the inhibition of apoplastic influx from the cortex toward the stele and symplastic Mn assimilation in the shoot protoplast, where the chloroplast is the main target (Lidon, 2001).

The distribution of Mn excess in both roots and shoots is dependent on plant species and genotype. Early research associated Mn tolerance in some plants with a greater retention of Mn excess in the roots, as mentioned by Andrew and Hegarty (1969) in regard to tropical and temperate legume species. The root retention of heavy metals has been attributed to the formation of metal complexes in roots (Foy *et al*., 1978). Metals with high electro-negativity

accumulate in roots in larger amounts than metals with low electro-negativity. In the latter instance, Mn and Zn metals can easily be translocated to the tops (Chino, 1981).

In proteomics studies, a comparison of Mn-sensitive and Mn-tolerant cultivars of cowpea (*Vigna unguiculata*.) has shown relevant features of leaf apoplast in the expression of Mn toxicity: formation of brown spots, induction of callose formation and an enhanced release of phenols and peroxidases into the apoplast (Fecht-Christoffers *et al*., 2003; Fecht-Christoffers *et al*., 2006). Specific proteins involved in the regulation processes, such as CO_2 fixation, stabilization of the Mn cluster of the photosystem II, pathogenesis-response reactions and protein degradation, were affected at low or high Mn levels, mainly in the Mn-sensitive cowpea cultivar (Führs *et al*., 2008). Chloroplastic proteins, which are important for CO_2 fixation and photosynthesis, were of lower abundance upon Mn-induced stress, suggesting the scavenging of metabolic energy for a specific stress response. Führs *et al*., (2008) concluded that a coordinated interplay of apoplastic and symplastic reactions seems to be important during the Mn-stress response in plants.

To alleviate metal toxicity in plants, the antioxidant systems are also considered an important tolerance mechanism. The antioxidant systems include antioxidant enzyme "scavengers" such as superoxide dismutase (SOD), catalase (CAT), peroxidases (phenol peroxidase, POX, ascorbate peroxidase, APX, guaiacol peroxidase, GPX) and the non-enzymatic antioxidant molecules: ascorbate, α-tocopherol, carotenoids, flavonoids and glutathione (Foyer and Noctor, 2003; Apel and Hirt, 2004). These constitute other mechanisms against ROS, produced by Mn toxicity. Higher activities of antioxidant enzymes are found in response to a Mn excess in woody plants (Lei *et al*., 2007), in herbs such as white clover (*Trifolium repens*.) and in ryegrass (*Lolium perenne*), which suggest a lower oxidative stress (Rosas *et al*., 2007; Mora *et al*., 2009). Another plant tolerance mechanism to metal toxicity is associated with lower metal uptake and translocation to other organs (Hall, 2002). The exudation of organic acid anions (carboxylates) to the rhizosphere may minimize the absorption by roots of such metals as aluminium and nickel (Ma *et al*., 2001; Yang *et al*., 1997). There are few reports regarding the release of organic acid anions by roots in the event of Mn toxicity (González and Lynch, 1999). Recently, Mora *et al*. (2009) reported that the root exudates of oxalate and citrate may decrease Mn availability in the rhizosphere, enhancing their Mn tolerance in ryegrass subjected to Mn toxicity.

Other nutrient applications can help minimize the effects of Mn toxicity. Thus, it has been reported that silica (Si) addition significantly decreases lipid peroxidation caused by an Mn excess decreasing the symptoms of Mn phytotoxicity and improving plant growth in some plants (Iwasaki *et al*., 2002; Shi *et al*., 2005). There is also an association between Mn toxicity and the decrease in Ca concentration in barley plants (*Hordeum vulgare*), indicating a competition and a specific interaction during the absorption and/or translocation of these elements (Alam *et al*., 2001). Another study, in which Ca is applied to reduce the Mn toxicity, showed that Ca additions inhibited Mn translocation from roots to shoots in barley plants, but did not affect the Mn absorption in roots (Alam *et al*., 2006). This suggested that Ca could avoid Mn accumulation in shoots, protecting the photosynthetic apparatus from the dangerous effect of an

excess of Mn. In barley, Mn toxicity could also be repressed by high K contents, which inhibit both the absorption and the translocation of Mn (Alam *et al*., 2005).

CONCLUSIONS AND PERSPECTIVES

Manganese is considered an essential micronutrient for the metabolic process in plants. Nevertheless, both deficiency and excess alter these processes. Acid soils make excessive Mn amounts toxic for the plants. Mn toxicity is a world-wide problem in areas with acid soils. This toxicity alters physiological, biochemical and molecular processes at the cell level. It is crucial to know the limitations of these soils for the purpose of soil-plant interaction management, especially in relation to the presence and Mn excess. Thus, the knowledge of Mn uptake, translocation, accumulation and resistance mechanisms in crop plants under Mn excess and toxicity is of great importance to crop improvement.

Most molecular and physiological approaches to Mn transport inside plant cells have recently been analyzed, as these are useful tools for understanding resistance mechanisms. However, the question as to which is the best candidate gene related to Mn toxicity in acid soil continues to elude plant scientists due to Mn toxicity in plants being a complex trait and involving multiple physiological and biochemical mechanisms and a wide array of genes. The understanding of these mechanisms will contribute to improving the yield and quality of cultivated plants in acid soils. Future efforts for developing Mn-tolerant plants should take all these aspects into account.

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RESEARCH PAPER

Excess manganese differentially inhibits photosystem I versus II in *Arabidopsis thaliana*

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Abstract

The effects of exposure to increasing manganese concentrations (50–1500 μM) from the start of the experiment on the functional performance of photosystem II (PSII) and photosystem I (PSI) and photosynthetic apparatus composition of *Arabidopsis thaliana* were compared. In agreement with earlier studies, excess Mn caused minimal changes in the PSII photochemical efficiency measured as F_v/F_m, although the characteristic peak temperature of the $S_{2/3}Q_B^-$ charge recombinations was shifted to lower temperatures at the highest Mn concentration. SDS-PAGE and immunoblot analyses also did not exhibit any significant change in the relative abundance of PSII-associated polypeptides: PSII reaction centre protein D1, Lhcb1 (major light-harvesting protein of LHCII complex), and PsbO (OEC33, a 33 kDa protein of the oxygen-evolving complex). In addition, the abundance of Rubisco also did not change with Mn treatments. However, plants grown under excess Mn exhibited increased susceptibility to PSII photoinhibition. In contrast, *in vivo* measurements of the redox transients of PSI reaction centre (P700) showed a considerable gradual decrease in the extent of P700 photooxidation ($P700^+$) under increased Mn concentrations compared to control. This was accompanied by a slower rate of $P700^+$ re-reduction indicating a downregulation of the PSI-dependent cyclic electron flow. The abundance of PSI reaction centre polypeptides (PsaA and PsaB) in plants under the highest Mn concentration was also significantly lower compared to the control. The results demonstrate for the first time that PSI is the major target of Mn toxicity within the photosynthetic apparatus of *Arabidopsis* plants. The possible involvement mechanisms of Mn toxicity targeting specifically PSI are discussed.

Key words: Chlorophyll fluorescence, Mn toxicity, photosystem I, PSI-associated proteins, PSII-associated proteins, redox state of P700.

Introduction

Manganese (Mn) is one of the most abundant metals in the Earth's crust and although it is an important essential micronutrient for all photosynthetic organisms can be also toxic when it is present in excess (Mukhopadhyay and Sharma, 1991; Marschner, 1995). Mn is considered the second most phytotoxic element, after aluminium (Al), affecting negatively the physiological and biochemical properties of plant species (Foy *et al.*, 1978, Foy, 1984; Millaleo *et al.*, 2010). An

excess of this metal occurs in acid soils with low pH (<5.5) and/or under reducing conditions (Marschner, 1995; Schaaf *et al.*, 2002), where Mn^{2+} is the predominant solution species and available ion to plant cells (Bradl, 2004). Thus, a Mn excess results in a sharp decrease in shoot height, biomass accumulation, and total leaf area of a woody species (*Populus cathayana*, Lei *et al.*, 2007), a reduction of the dry weights (DW) of both shoots and roots in ryegrass cultivars (*Lolium perenne*, Mora *et al.*, 2009), and *Trifolium repens* (Rosas *et al.*, 2007). Furthermore, excess Mn can result in oxidative stress as indicated by the accumulation of H_2O_2 (Demirevska-Kepova *et al.*, 2004; Lei *et al.*, 2007), high levels of apoplastic H_2O_2-consuming peroxidases (Fecht-Christoffers *et al.*, 2003), and high level of lipid peroxidation (Mora *et al.*, 2009). Mn stress induced an enhancement of antioxidant enzyme activity in leaves of legumes (González *et al.*, 1998), and it was also demonstrated in perennial ryegrass (Mora *et al.*, 2009) and woody species (Lei *et al.*, 2007). More recently, a proteomic and transcriptomic studies have demonstrated that chloroplastic proteins important for CO_2 fixation and photosynthesis were of lower abundance upon Mn stress of cowpea (Führs *et al.*, 2008).

Mn has an important role in both the structure and functions of the photosynthetic apparatus (Mukhopadhyay and Sharma, 1991). Mn is a constitutive element associated with the oxygen-evolving complex of photosystem II (PSII), an important multiprotein pigment complex embedded in the thylakoid membranes (Hankamer *et al.*, 1997; Enami *et al.*, 2008). Therefore, the Mn cluster, together with other ions and extrinsic proteins that constitute the oxygen-evolving complex, is required to oxidize water and reduce P680, the reaction centre of PSII (Kern and Renger, 2007; Ferreira *et al.*, 2004; Rutherford and Boussac, 2004). In conjunction with photosystem I (PSI) and linear electron transport, these reducing equivalents (electrons) are used primarily in the conversion of CO_2 into carbohydrate (Ferreira *et al.*, 2004). In addition, Mn is indispensable as a cofactor for various enzymes involved in redox reactions such as Mn-superoxide dismutase, an essential enzyme involved in protection against oxidative stress in plants (Burnell, 1988; Bowler *et al.*, 1994).

A number of studies have suggested that chloroplasts and photosynthesis are the major targets of Mn toxicity. Indeed, increased amounts of Mn have been reported for chloroplasts isolated from Mn-stressed common bean (González and Lynch, 1999) and rice leaves (Lidon *et al.*, 2004). Distinctive ultrastructural changes showing swelling of granal and stromal thylakoids have been also observed in the chloroplasts of *Citrus volkameriana* (Papadakis *et al.*, 2007) and maize plants (Doncheva *et al.*, 2009) under Mn excess. It has been demonstrated that high Mn accumulation is associated with inhibition of the net photosynthesis and carboxylation efficiency in various plant species. The decline of photosynthesis is considered as one of the major mechanisms constituting the toxic effects of excess Mn and is proposed as an early indicator for Mn toxicity in tobacco (Nable *et al.*, 1988), rice (Lidon *et al.*, 2004) and wheat (Macfie and Taylor, 1992). Reduced CO_2 assimilation induced by excess Mn was also reported for common bean (González and Lynch, 1997), deciduous broad leaved trees (Kitao *et al.*, 1997a), and seedlings of *Citrus grandis* (Li *et al.*, 2010). Interestingly, the maximum photochemical efficiency of PSII (F_v/F_m) was not substantially affected by Mn accumulation in various plant species over a wide range of leaf Mn concentrations (Nable *et al.*, 1988; Kitao *et al.*, 1997b; Subrahmanyam and Rathore, 2000; Hajiboland and Hasani, 2007; Doncheva *et al.*, 2009).

The reduction in photosynthesis by excess leaf Mn has been generally attributed to modification of ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco; Ohki, 1984; Houtz *et al.*, 1988; McDaniel and Toman, 1994; Kitao *et al.*, 1997b). It has been demonstrated that a high level of Mn affects primarily the activity rather than the amount of Rubisco (Houtz *et al.*, 1988; Chatterjee *et al.*, 1994) and the presence of excess Mn induces enhanced oxygenase activity (Jordan and Ogren, 1983). In addition, the decline of photosynthesis under Mn stress conditions was also ascribed to peroxidative impairment of photosynthetic enzyme activities caused by polyphenol oxidation products (Vaughn and Duke, 1984; Panda *et al.*, 1987).

In spite of these studies, the mechanisms of Mn toxicity causing a decrease in CO_2 assimilation are still not well understood. In addition, very limited information concerning the toxic effect(s) of excess Mn on the polypeptide composition of both PSII and, especially, PSI is available. Therefore, the objective of this study was to evaluate the role of specific Mn-induced changes in the structure and function of PSII and PSI, which could help to understand the mechanisms by which Mn excess may cause a decrease of CO_2 assimilation in *Arabidopsis thaliana.*

Materials and methods

Plant material and growth conditions

Seeds of *A. thaliana* (wild type Columbia) were germinated in a substrate mix (82.5% sphagnum peat moss, 12.5% perlite, 5% vermiculite-Pro-Mix, Premier Tech Horticulture) in controlled environment growth cabinets (model GCW15, Environmental Growth Chambers, Chagrin Falls, OH, USA) with a photosynthetical active radiation (PAR) of 250 µmol photons m^{-2} s^{-1}, 20/20 °C day/night temperatures, 50% relative humidity, and 8/16 light/dark cycle to prevent flowering. Water was supplied every 5 days. After 15 days, seedlings were transplanted separately in pots with vermiculite and placed in trays. Each tray containing seven pots (one plant per pot) were supplied with Hoagland nutrient solution for 2 weeks before applying the Mn treatments.

Manganese treatments

Manganese treatments included the final concentrations: 18 (control), 50, 500, 1000, and 1500 µM Mn according to Delhaize *et al.* (2007). Manganese was applied as $MnCl_2.4H_2O$. Control plants exposed to 18 µM Mn as the optimal dose for Mn for *Arabidopsis* (Cailliatte *et al.*, 2010). The five Mn treatments were grown in five labelled trays, with 500 ml of Hoagland's solution. The trays were maintained in controlled environment growth chambers under the same conditions described above. The pH was adjusted to 5.3 with diluted HCl daily and nutrient solution was changed every 5 days. Plants were subjected to these treatments for 21 days before harvest.

Plant growth measurements

Prior to beginning the Mn treatments, three plant samples were dried in a forced-air oven (70 °C, 48 h) and weighed to determine dry weight

(W1) at day 0. Similarly, at the end of the experiment, plants were harvested and collected for dry weight measurements (W2). These data were used to determine mean relative growth rate (RGR) according to Fernando *et al.* (2009):

$$RGR = \frac{(\ln W2 - \ln W1)}{t2 - t1}$$

as g DW d^{-1}.

Manganese concentration

For Mn chemical analysis, samples of shoot and roots were dry ashed in a muffle furnace at 500 °C for 8 h and digested with 2 M HCl. Manganese was extracted as described by Sadzawka *et al.* (2004), and the Mn concentration was determined using a simultaneous multi-element atomic absorption spectrophotometer (model 969, Unicam, Cambridge, UK).

Thylakoid preparation, SDS-PAGE, and immunoblotting

Thylakoid membranes for SDS-PAGE were isolated as described earlier (Krol *et al.*, 1999). Leaf material was ground in cold isolation buffer (50 mM Tricine, 0.4 M sorbitol, 10 mM NaCl, 5 mM $MgCl_2$, pH 7.8) in a mortar on ice, filtered through two layers of miracloth (typical pore size 22–25 µm; Calbiochem), and centrifuged for 15 min (10,000 *g*). The supernatant was removed and the pellet was resuspended in cold isolation buffer. Total chlorophyll concentration was measured in 90% (v/v) acetone (Arnon, 1949). For immunodetection of Rubisco, total leaf proteins were extracted as described in Rosso *et al.* (2009). Protein content was measured using a BCA protein assay kit (Pierce) by following the absorbance at 562 nm using a spectrophotometer (DU-640, Beckman Coulter). Proteins were separated by SDS-PAGE according to Laemmli (1970), using 15% (w/v) polyacrylamide gel in the presence of 6 M urea in the separating gel. Chloroplast thylakoids were solubilized with SDS (SDS:chlorophyll 20:1) and 15 µg chlorophyll was loaded per lane. All samples for separation of total proteins were loaded on an equal protein basis of 20 µg protein per lane (Rosso *et al.* 2009). Immunoblotting was performed by electrophoretically transferring the proteins from SDS:PAGE gel to nitrocellulose membrane (Bio-Rad) according to the method of Towbin *et al.* (1979). Proteins were probed with antibodies (AgriSera AB, Vanas, Sweden) raised against the reaction centre polypeptides of PSI: PsaA, PsaB (1:2000), the major light-harvesting protein of PSII complex (LHCII) Lhcb1 protein (1:5000), the PSII oxygen-evolving complex extrinsic protein PsbO (33 kDa, 1:2000), the PSII reaction centre protein: D1 and Rubisco (1:5000). As secondary antibodies, goat anti-rabbit IgG conjugated with horseradish peroxidase (Sigma-Aldrich) were used. Polypeptides were detected using enhanced chemiluminescence detection kit (Amersham Biosciences) and visualized by exposing the membrane to X-ray film. Densitometric scanning and analysis of X-ray films from each replicate immunoblot was performed with a Hewlett Packard ScanJet 4200C desktop scanner and ImageJ 1.41o densitometry software (Wayne Rosband, National Institute of Health, USA, http://rsbweb.nih.gov/ij).

Measurement of the redox state of P_{700}

The redox state of P_{700} was determined *in vivo*, in dark-adapted (20 min) *Arabidopsis* leaves under growth temperature and ambient O_2 and CO_2 conditions using a PAM-101 modulated fluorometer equipped with a dual-wavelength emitter-detector ED-P700DW unit and PAM-102 units (Klughammer and Schreiber, 1991) as described in detail by Ivanov *et al.* (1998). Far-red light (λ_{max}=715 nm, 10 W m^{-2}, Schott filter RG 715) was provided by an FL-101 light source. The redox state of P700 was evaluated as the absorbance change around 820 nm ($\Delta A_{820-860}$) in a custom-designed cuvette. Multiple turnover (MT, 50 ms) and single turnover (ST, half peak 14 µs) saturating flashes were applied with XMT-103 and XST-103 (Walz) power/control units, respectively. The relative functional pool size of intersystem electrons on a P_{700} reaction centre basis was calculated as the complementary area between the oxidation curve of P700 after either ST or MT pulse excitation (ST and MT areas) and the stationary level of P700 under far-red excitation (Asada *et al.*, 1993; Ivanov *et al.*, 1998). Capacity of PSI cyclic electron (e^-) flow was determined as the half time for the dark decay of the P700 signal (Ivanov *et al.*, 1998).

Modulated chlorophyll fluorescence measurements

Modulated imaging fluorometer (IMAGING-PAM, Heinz Walz, Efeltrich, Germany) was used for capturing the chlorophyll fluorescence images and estimation of the maximal photochemical efficiency of PSII [$F_v/F_m = (F_m - F_o)/F_m$], effective photochemical efficiency of PSII (Φ_{PSII}), photochemical (qP), and non-photochemical (NPQ) fluorescence quenching parameters using the nomenclature of van Kooten and Snel (1990) as described earlier (Ivanov *et al.*, 2006a). Control and Mn-treated *Arabidopsis* plants were dark adapted (20 min) and all chlorophyll fluorescence measurements were performed *in vivo* at room temperature. Fluorescence images were captured by a CCD camera (IMAG-K, Allied Vision Technologies) featuring a 640 × 480 pixel CCD chip size and CCTV camera lens (Cosmicar/Pentax F1.2, f = 12 mm). Light emitting diode ring array (IMAG-L) consisting of 96 blue LEDs (470 nm) provided standard modulated excitation intensity of 0.5 µmol quanta m^{-2} s^{-1} (modulation frequency 1–8 Hz) for measuring the basal (F_0) chlorophyll fluorescence and a saturation pulse of 2400 µmol quanta m^{-2} s^{-1} PAR for measuring the maximal chlorophyll fluorescence (F_m). The maximal photochemical efficiency of PSII (F_v/F_m) was determined as ($F_m - F_0$)/ Fm. The effective photochemical efficiency of PSII (Φ_{PSII}) was calculated from the expression ($F'_m - F_s$)/F'_m (Genty *et al.*, 1989), photochemical quenching (qP) was calculated as ($F'_m - F_s$)/($F'_m - F'_0$) (Schreiber *et al.*, 1994), electron transport rate (ETR) was calculated as PAR × 0.5ΦPSII × 0.84 (Genty *et al.*, 1989), and regulated non-photochemical quenching (Φ_{NPQ}) and constitutive photochemical quenching (Φ_{NO}) was determined according to Kramer *et al.* (2004). All measurements were performed at 0, 5, 10, 15, and 21 days in plants subjected to the different Mn treatments.

At the end of experiments (21 days), leaves of *Arabidopsis* plants growing under the different Mn treatments and 250 µmol m^{-2} s^{-1} PAR (normal light) were cut and exposed to high light (1000 µmol s^{-1} m^{-2}, PAR) at 1, 2, and 4 hours. During each time of exposition, F_v/F_m was determined.

Thermoluminescence measurements

Thermoluminescence (TL) measurements of control and Mn-treated *Arabidopsis* leaves were performed on a personal-computer-based TL data acquisition and analysis system as described earlier (Ivanov *et al.*, 2001, 2006b). A photomultiplier tube (Hamamatsu R943-02, Hamamatsu Photonics, Shizuoka-ken, Japan) equipped with a photomultiplier power supply (model PS-302, EG&G Electro Optics), and a preamplifier (model C1556-03) was used as a radiation measuring set. The heating rate was 0.6 °C s^{-1}. For identifying the $S_{2/3}Q_B^-$ charge recombination peaks, dark-adapted leaf discs were subjected to two consecutive saturating microsecond flashes of white light (1.5 µs peak width at 50% of maximum) applied by a xenon-discharge flash lamp (XST103, Heinz Walz). Dark-adapted leaves (20 minutes at 20 °C) were cooled to 2 °C prior to exposing to the flashes. The nomenclature of Sane *et al.* (2012) was used for characterization of the TL glow peaks.

Experimental design and statistical analysis

The experimental design is a randomized split design, with 1 species × 5 Mn treatments × 7 replicates for the physiological determinations and 1 species × 5 Mn treatments × 3 replicates × 4 times (0, 1, 2, and 4 hours) for the photoinhibition measurements. Data correspond to the means of replicates for each determination as indicated above. Data were tested in their normality and equal variance by the Shapiro–Wilk test and then data were analysed by a one-way ANOVA. Significant differences between means were established by using the multiple comparisons test

of Tukey's ($P < 0.05$). All analyses were performed with Sigma Stat 2.0 software (SPSS), where differences between the values were considered significant at $P \leq 0.05$.

Results

A statistically significant decrease in total biomass of leaves treated with 500 and 1500 µM Mn concentrations was found compared to the control plants (18 µM Mn), although a decrease of the total leaf biomass was found at all Mn concentrations used. In contrast, root biomass exhibited significant decrease in all treatments ($P < 0.05$, Fig. 1). It should be noted, however, that while the decrease of leaf biomass was only 25% lower at the highest Mn concentration used (1500 µM), the biomass of roots was more affected and demonstrated a 2.5-fold decrease even at the lowest Mn concentration tested (50 µM). This is consistent with the RGR data, where a considerably higher reduction of root RGR was observed compared to leaves across the Mn treatments relative to control plants ($P < 0.05$, Table 1). In addition, visual symptoms of Mn toxicity (chlorosis) in leaves were observed predominantly in the highest Mn treatment (data not shown).

Table 1. Relative growth rates of *Arabidopsis thaliana* (leaves and roots) growing at increased Mn treatments. Plants were grown under acidic conditions (pH 5.3) for 21 d. Different lower-case letters indicate statistically significant differences between the Mn treatments ($P < 0.05$).

Mn treatment (µM)	Relative growth rate (g DW d^{-1})	
	Leaves	Roots
Control	0.069 ± 0.001^{a}	0.206 ± 0.006^{a}
50	0.068 ± 0.003^{a}	0.071 ± 0.005^{b}
500	0.046 ± 0.006^{b}	0.053 ± 0.006^{b}
1000	$0.059 \pm 0.003^{a,b}$	0.064 ± 0.007^{b}
1500	0.040 ± 0.004^{b}	0.057 ± 0.004^{b}

Analysis of the total Mn amount in plants exposed to increasing Mn concentrations have demonstrated a gradual increase of Mn in leaves and roots (Fig. 2). However, while Mn concentrations of roots reached values up to ~8100 mg kg^{-1} Mn at the highest Mn treatment, which represents a 40-fold increase of Mn, the increased accumulation of Mn in leaves was much lower (15-fold) compared to roots (Fig. 2). Thus, the differential effects of Mn treatments on the total

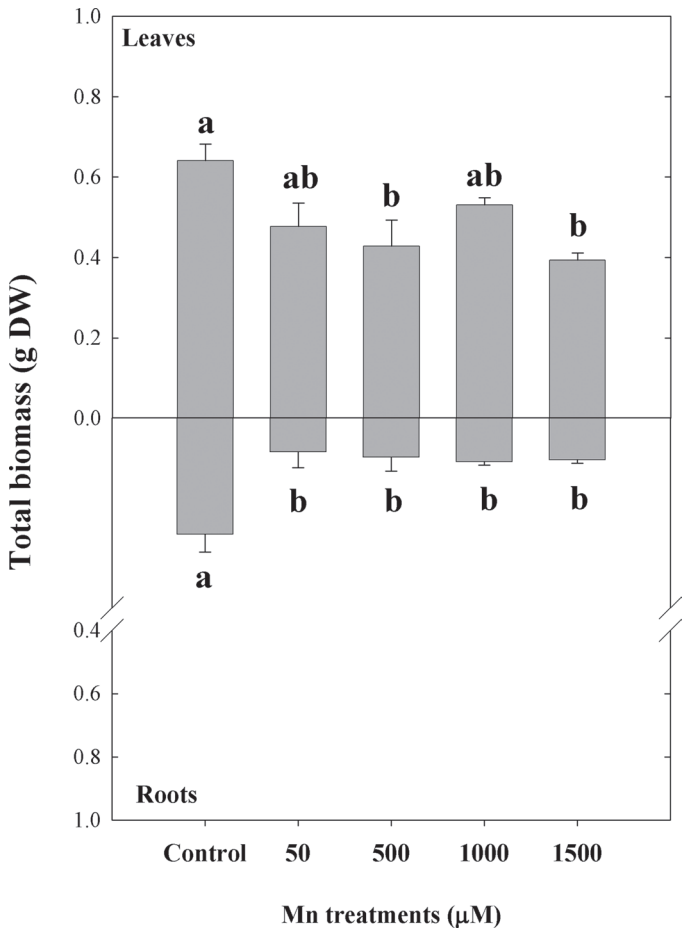

Fig. 1. Total biomass (leaves and roots) of *Arabidopsis thaliana* plants subjected to Mn treatments at the end of experiment (21 days). Different lower-case letters indicate significant differences between the Mn treatments ($P \leq 0.05$).

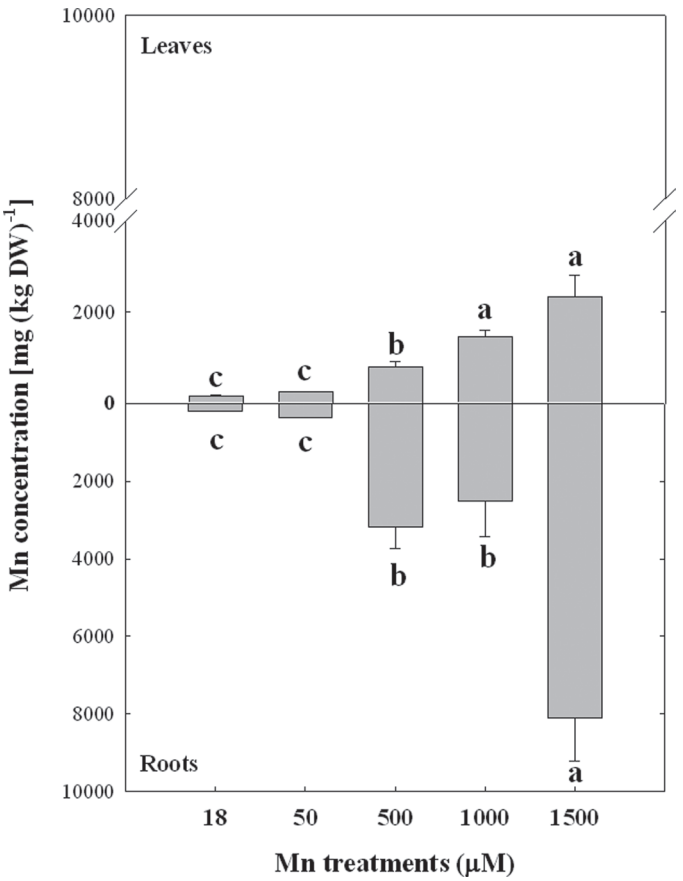

Fig. 2. Mn concentration in leaves and roots of *Arabidopsis thaliana* plants subjected to Mn treatments at the end of experiment (21 days). Different lower-case letters indicate statistically significant differences between the Mn treatments ($P \leq 0.05$).

Table 2. Maximum photochemical efficiency of PSII (F_v/F_m) in leaves of *Arabidopsis thaliana* measured at 0, 5, 10, 15 and 21 days subjected to increasing Mn treatments. Results are means ± SE of five replicates of two plants each. Different lower-case letters indicate significant differences between the Mn treatments at the same time, while different upper-case letters indicate significant differences between the times at a same Mn treatment ($P < 0.05$).

Mn treatment (μM)	F_v/F_m				
	0 days	5 days	10 days	15 days	21 days
Control	0.803^{aA}	0.803^{aA}	0.803^{aA}	0.803^{aA}	0.803^{aA}
50	0.806^{aA}	0.799^{aA}	0.800^{aA}	0.800^{aA}	0.800^{aA}
500	0.803^{aA}	0.801^{aA}	0.804^{aA}	0.787^{bA}	0.791^{aA}
1000	0.797^{aA}	0.805^{aA}	0.800^{aA}	0.778^{bB}	0.773^{bB}
1500	0.802^{aA}	0.798^{aA}	0.775^{bB}	0.769^{bcBC}	0.756^{cC}

biomass and RGR in leaves and roots could be explained by the higher accumulation of Mn in roots.

The effects of exposure to increasing Mn concentrations on the maximum photochemical efficiency of PSII measured as F_v/F_m in *Arabidopsis* leaves are presented in Table 2. A time-course measurement did not reveal any statistically significant changes of F_v/F_m values at the lower Mn doses (50 and 500 μM) for the entire period of treatment compared to control plants (18 μM Mn). At higher Mn concentrations (1000 and 1500 μM), a small reduction (6%) of F_v/F_m was observed only at a later stage (days 15 and 21) of treatment, the statistically significant ($P < 0.05$) differences being registered at the harvest time (21 days, Table 2). Moreover, minimal Mn-induced changes were detected in the light response curves of photochemical quenching (qP), effective photochemical efficiency of PSII (Φ_{PSII}), and PSII electron transport rates (ETR) even after 21 days of Mn treatments with the highest dose tested (1500 μM, data not shown).

In addition, TL measurements were used as an alternative approach for assessing the effects of Mn excess on the photosynthetic PSII-associated electron transfer reactions especially at its reducing side (Vass and Govindjee, 1996; Sane *et al.*, 2012). Since most of the photosynthetic TL components have been assigned to arise from the reversal of light-driven charge separation in PSII, TL properties of photosynthetic apparatus provide information on the activation energies associated with the back reactions of electron acceptors (Q_A and Q_B) with the electron donors (S_2 and S_3) of PSII (Vass and Govindjee, 1996; Sane, 2004; Sane *et al.*, 2012). The temperature maxima (T_M) of the TL peaks related to the recombination of these charge pairs reflect the activation energies and hence a measure of the redox potentials of the participating oxidized and reduced donors (de Vault and Govindjee, 1990). Typical TL glow curves representing $S_{2/3}Q_B^-$ charge recombinations of control and Mn-treated *Arabidopsis* plants obtained following excitation with two consecutive saturating flashes are shown in Fig. 3. The experimental data summarized in Table 3 indicate that treatment with a Mn dose of 500 μM did not exhibit significant differences of the TL

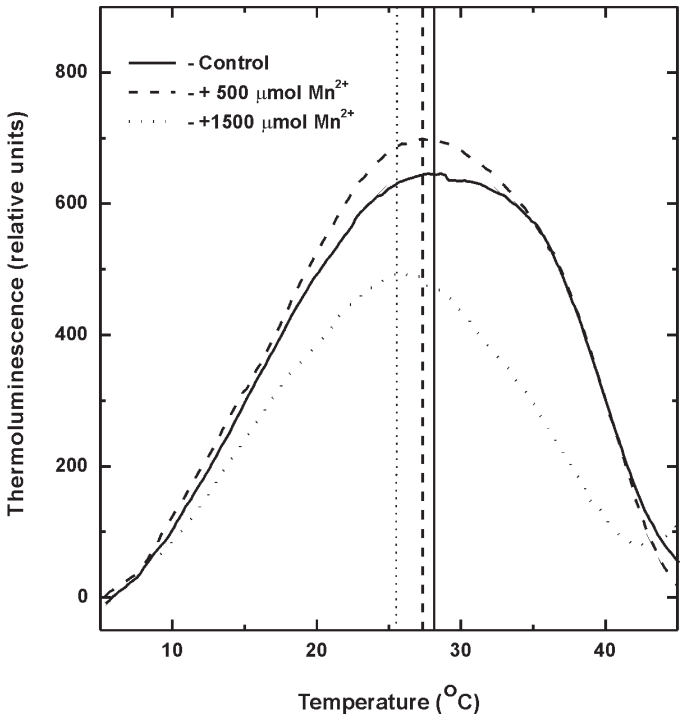

Fig. 3. Thermoluminescence (TL) glow curves of $S_{2/3}Q_B^-$ charge recombinations in control (solid lines) and Mn-treated (dashed lines, 500 μM Mn^{2+}; dotted lines, 1500 μM Mn^{2+}) *Arabidopsis* leaves after illumination with two single turnover flashes. TL glow curves were recorded immediately after illumination. The presented glow curves are averages from four independent measurements.

peak position, while treatment with the highest concentration (1500 μM) induced a low temperature shift of the T_M to 25.8 °C compared to control plants. Besides this, the amplitudes and the integrated areas of the TL peaks representing the $S_{2/3}Q_B^-$ charge recombination used for assessing the PSII photochemistry was not affected at 500 μM Mn excess, but the overall TL yield was significantly reduced (45%) in plants treated with 1500 μM Mn compared to controls (Table 3).

To further test whether this small but significant Mn-induced effect on PSII photochemistry had physiological implications under additional stress conditions, *Arabidopsis*

Table 3. Characteristic thermoluminescence peak emission temperatures (T_M) and the overall TL emission area (A) of $S_{2/3}Q_B^-$ glow peaks of control and Mn-treated (21 days) *Arabidopsis* plants. The samples (leaf disks) were dark adapted for 30 min then cooled to 2 °C and subsequently illuminated with two single turnover flashes of white light. The peak areas are presented as a percentage of the total thermoluminescence light emission in control leaves. Values are mean ± SE calculated from four independent experiments.

Mn treatment (μM)	T_M (°C)	A (%)
Control	28.7 ± 1.1	100.0
500	29.1 ± 1.3	106.2 ± 7.6
1500	25.8 ± 0.9	54.5 ± 7.9

Table 4. Effect of high light treatments (photon flux density 1000 µmol photons m^{-2} s^{-1} for 1, 2, and 4 hours) on the maximal photochemical efficiency of PSII measured as F_v/F_m in control *Arabidopsis thaliana* leaves and plants exposed for 21 days to different Mn doses. Results are mean ± SE of five repetitions in three leaves of two plants each. Time 0 was measured in plants subjected to 250 µmol m^{-2} s^{-1} photon flux density. Different upper-case letters indicate significant differences between the times of exposure at the same Mn treatment ($P < 0.05$).

Irradiance exposition time (h)	F_v/F_m		
	Control	500 µM Mn	1500 µM Mn
0	0.815 ± 0.003^A	0.812 ± 0.004^A	0.780 ± 0.014^A
1	0.761 ± 0.012^A	0.750 ± 0.002^A	0.677 ± 0.014^B
2	0.752 ± 0.007^A	0.731 ± 0.002^A	0.619 ± 0.028^B
4	0.685 ± 0.036^A	0.689 ± 0.004^A	0.388 ± 0.042^C

leaves subjected to different Mn treatments for 21 days were exposed to high light stress (Table 4). Indeed, the photoinhibitory effect on PSII, measured as a decrease in F_v/F_m, was much stronger (49%) at the highest Mn treatment (1500 µM) compared to control plants, and 500 µM Mn treated plants exhibited only a 15% decrease in F_v/F_m values after 4 h of exposure to high light (Table 4).

The extent of far-red light-induced absorbance decrease at 820 nm ($\Delta A_{820-860}$) of *Arabidopsis* leaves (Klughammer and Schreiber, 1991; Ivanov *et al.*, 1998, 2006a) was used to estimate the potential functional differences of PSI and photosynthetic electron transport pathways between plants exposed to different Mn treatments. Typical traces representing *in vivo* measurements of oxidation–reduction transients of P_{700} in control and Mn-treated plants are shown in Fig. 4. The relative amount of $P700^+$, measured as $\Delta A_{820-860}$, gradually decreased with increasing Mn concentrations and was 30% lower in Mn-treated plants at the highest concentration used (1500 µM, Fig. 4 and Table 5). Concomitantly, kinetic measurements of dark re-reduction of $P700^+$ after turning off the far-red light, which is thought to reflect the extent and/or capacity for cyclic electron flow around PSI (Maxwell and Biggins, 1976; Ravenel *et al.*, 1994), indicated significantly slower (46%) re-reduction of $P700^+$ in Mn (1500 µM)-treated plants compared to control plants (Table 5). In addition, the apparent electron donor pool size to PSI (e^-/P700) estimated by measuring single-and multiple-turnover flash-induced $\Delta A_{820-860}$ under steady-state oxidation of PSI by far-red light (Asada *et al.*, 1993; Ivanov *et al.*, 1998) demonstrated a significant decrease in Mn-treated plants (Table 5). This indicates that the pool size of electrons that can be donated to photooxidized P700 ($P700^+$) from the stroma in control plants was 37% higher compared to plants treated with the highest Mn dose (13 electrons per P700, Table 4).

The major photosynthetic components within the thylakoid membranes of control and Mn-treated *Arabidopsis* plants were compared by SDS-PAGE and immunodetection to quantify their relative abundance. Immunoblot analyses did not exhibit any significant Mn-stress-induced changes

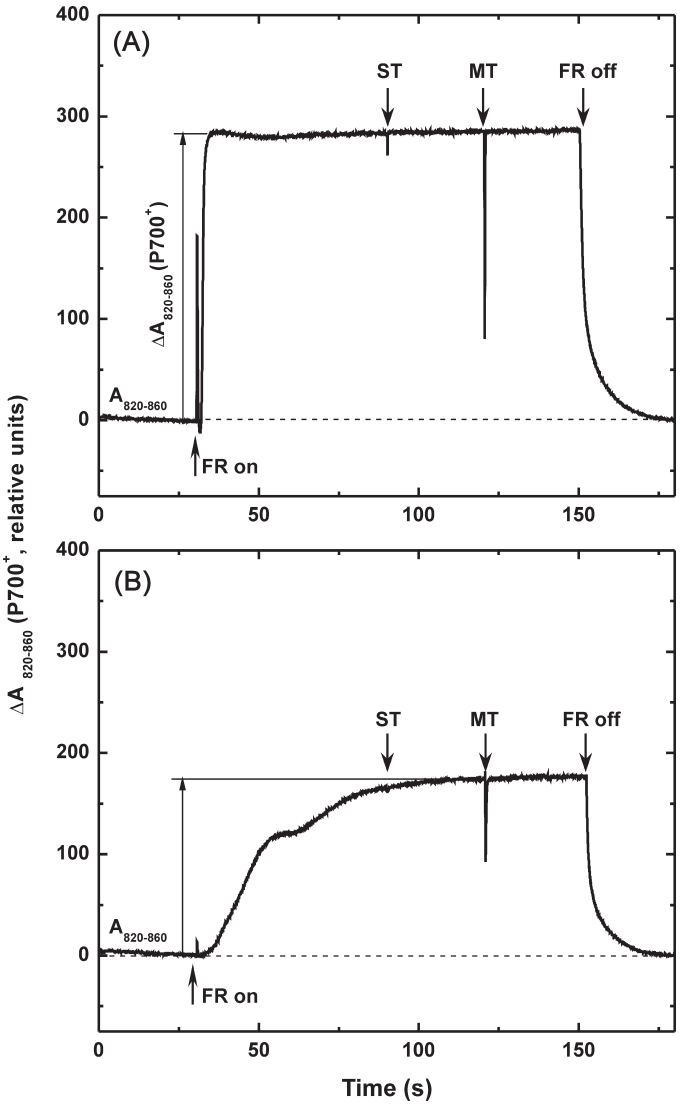

Fig. 4. Typical traces of *in vivo* measurements of P700 oxidation by far-red light (FR) in control (A, 18 µM Mn) and Mn-treated (B, 1500 µM Mn) *Arabidopsis* plants. After reaching a steady state level of $P700^+$ by FR light, single turnover (ST) and multiple turnover (MT) pulses of white light were applied. Arrows indicate application of ST, MT, and FR light sources. The measurements were performed at the growth temperature of 20 °C.

in the relative abundance of PSII-associated polypeptides, as revealed by the densitometry analysis of the immunoblot bands for D1 (the PSII reaction centre protein), Lhcb1 (major light-harvesting protein of LHCII), and PsbO (extrinsic protein of the oxygen-evolving complex (Fig. 5). The abundance of Rubisco was only marginally affected by the Mn treatments. In contrast, the abundance of reaction centre polypeptides of PSI (PsaA and PsaB) was significantly reduced in Mn-treated compared with control thylakoids (Fig. 5A). The densitometric analysis demonstrated that the relative abundance of PsaA and PsaB in Mn-treated *Arabidopsis* was only about 20 and 60%, respectively, of that observed in the control plants (Fig. 5B). Thus, the quantitative analysis of photosynthetic polypeptides clearly indicates that excess Mn has

Table 5. Effects of Mn treatments on far-red light-induced steady state oxidation of P700 ($\Delta A_{820-860}$, $P700^+$), the relative intersystem electron donor pool size to PSI (e^- /P700) and half times for $P700^+$ reduction ($t_{1/2}$) of *Arabidopsis* leaves at 21 days of treatment. Different lower-case letters indicate significant differences between the Mn treatments ($P < 0.05$). MT, multiple turnover; ST, single turnover.

Mn treatment (μM)	$\Delta A_{820-A860}$ ($P700^+$)	e^-/P700 (MT_{area}/ST_{area})	$t_{1/2}$ (s)
Control	423.2 ± 7.9^a	20.7 ± 0.9^a	0.670 ± 0.05^b
50	416.4 ± 8.3^a	15.7 ± 0.6^b	$0.812 \pm 0.06^{a,b}$
500	368.4 ± 8.3^b	16.4 ± 0.6^b	$0.938 \pm 0.08^{a,b}$
1000	293.4 ± 8.4^c	15.6 ± 0.5^b	$0.908 \pm 0.07^{a,b}$
1500	292.9 ± 4.2^c	13.0 ± 0.3^c	0.984 ± 0.08^a

a greater effect on PSI-associated proteins rather than PSII. Consequently, the differences in the extent of P700 oxidation ($P700^+$) and the kinetics of $P700^+$ reduction between control and Mn-treated plants are consistent with the lower levels of PSI-associated immundetectable PsaA and PsaB protein complexes.

Discussion

In agreement with a number of previous studies examining the effects of Mn stress on various plant species (Alam *et al.*, 2006; Lei *et al.*, 2007; Doncheva *et al.*, 2009; Mora *et al.*, 2009; Stoyanova *et al.*, 2009; Khabaz-Saberi *et al.*, 2010) *Arabidopsis* plants subjected to increasing Mn concentrations, also exhibited a reduction in dry weight of both shoots and roots at doses between 500 and 1500 μM Mn (Fig. 1). The decline in biomass corresponded with a gradual increase of Mn concentrations in both shoot and roots of *Arabidopsis* subjected to excess Mn supply (Fig. 2). It should be noted that the decline of biomass was more pronounced in roots, where even at the lowest Mn treatment (50 μM) the dry weight was 2.5-fold lower compared to leaves. Similar results have been reported by Delhaize *et al.* (2007), where transporter proteins were implicated in the endogenous Mn tolerance of wild-type *Arabidopsis*. More recently, Mora *et al.* (2009) demonstrated that Mn-tolerant ryegrass cultivars accumulated higher Mn concentrations in roots than shoots, while Mn-sensitive cultivars exhibited a greater Mn translocation from roots to shoots. These results are also consistent with studies in legumes such as white clover (*T. repens* L., Rosas *et al.*, 2007). However, in two contrasting populations of *P. cathayana*, acclimated to wet and dry climate exposure to excess Mn caused an increase in Mn content of plant tissues especially in leaves and a visual symptoms of Mn toxicity (chlorosis) at high Mn concentrations (Lei *et al.*, 2007). The chlorosis observed in the present experiments (data not shown) also correspond to a decreased amounts of both Chl a and Chl b could be due to a higher Mn accumulation in leaves after exposure to Mn excess, thus suggesting a damage to the photosynthetic apparatus as reported by Demirevska-Kepova *et al.* (2004).

Interestingly, while reduced CO_2 assimilation induced by excess Mn has been reported in many species and is considered one of the major physiological effects of Mn toxicity (Nable *et al.*, 1988; Macfie and Taylor, 1992; González and Lynch, 1997; Kitao *et al.*, 1997a; Lidon *et al.*, 2004; Li *et al.*, 2010), the functional integrity of the photosynthetic apparatus assessed by the maximum photochemical efficiency of PSII (F_v/F_m) did not decline as a result of exposure to excess Mn in various plant species (Nable *et al.*, 1988; Kitao *et al.*, 1997b; Subrahmanyam and Rathore, 2000; Hajiboland and Hasani, 2007; Doncheva *et al.*, 2009). However, some studies have reported a substantial decrease in F_v/F_m as a result of excess Mn treatment in *Citrus* species (Papadakis *et al.*, 2007; Li *et al.*, 2010), rice (Lidon *et al.*, 2004), Mn-sensitive maize (Doncheva *et al.*, 2009), and cucumber (Feng *et al.*, 2009). Furthermore, Kitao *et al.* (1997b) have shown that the potential maximum photochemical efficiency of PSII (F_v/F_m) is not affected by excess Mn in white birch, although the reduction state of PSII primary electron acceptor (Q_A) was increased at high Mn concentrations. These results clearly indicate that the excess Mn-induced decline in CO_2 assimilation may or may not be accompanied by changes in PSII photochemistry and this response is species dependent. The experimental data presented in this study also failed to demonstrate any substantial effects of excess Mn within a wide range of Mn concentrations on the maximum photochemical efficiency of PSII in *Arabidopsis* plants (Table 2). Assessing the relative abundance of PSII-associated proteins also showed no changes in the immunodetectable amounts of PSII reaction centre protein D1, the light-harvesting chlorophyll-protein complex of PSII (Lhcb1), and the manganese-stabilizing 33-kDa protein of the water splitting complex of PSII (PsbO) polypeptides (Fig. 5) in plants exposed to high Mn concentrations compared to control *Arabidopsis*. However, the observed low temperature shift of the $S_{2/3}Q_B^-$ charge recombination and much lower overall TL emission implies lower redox potential of Q_B (Fig. 3 and Table 3) confirms the suggestion of altered reduction state of PSII acceptor side in Mn-stressed plants (Kitao *et al.*, 1997b). Since Q_A is in quasi-equilibrium with Q_B and the PQ pool, the present results imply that lowering the redox potential of Q_B will decrease the probability for forward electron transfer between the two quinone acceptors by shifting the redox equilibrium between $Q_A^-Q_B$ and $Q_AQ_B^-$ towards $Q_A^-Q_B$ (Minagawa *et al.*, 1999; Ivanov *et al.*, 2002, 2003) in plants exposed to high Mn concentrations.

In addition to the lack of significant inhibitory effects of excess Mn on PSII photochemistry discussed above, an earlier study reported that the photochemistry of photosystem I (PSI) and the photosynthetic electron transport were not significantly affected during early development of Mn toxicity in tobacco plants (Nable *et al.*, 1988). However, a decreased Hill activity in isolated chloroplasts was found in mungbean leaves exposed to toxic Mn concentrations (Sinha *et al.*, 2002). More recently, Li *et al.* (2010) have suggested that Mn excess can effectively impair the whole photosynthetic electron transport chain, thus restricting the production of reducing equivalents and limiting the rate of CO_2 assimilation in *Citrus grandis* seedlings. Despite these few studies,

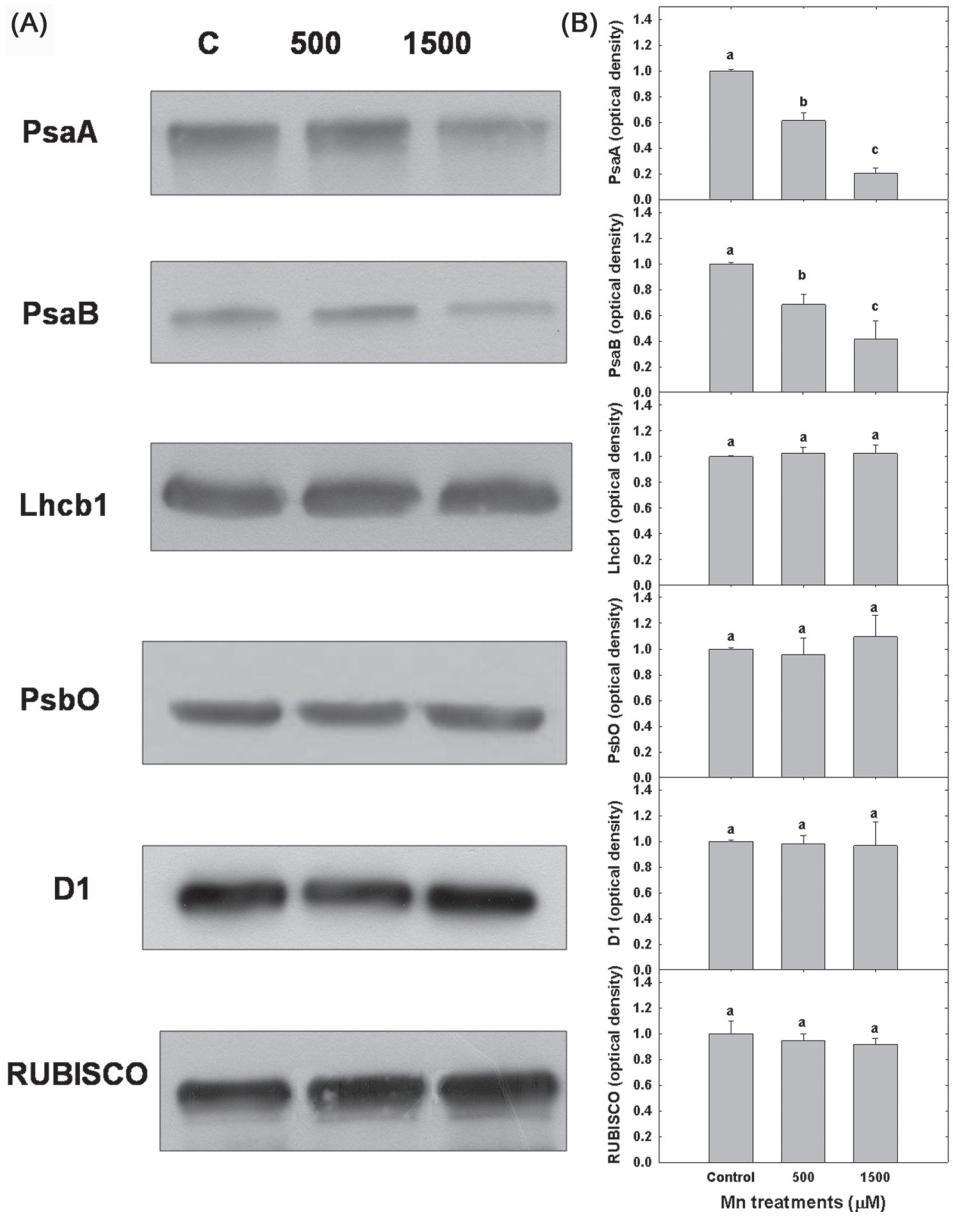

Fig. 5. (A) Representative immunoblots of SDS-PAGE separated proteins from thylakoid membranes probed with antibodies raised against PSI (PsaA and PsaB) and PSII (D1, PsbO, Lhcb1)-associated polypeptides and Rubisco (PbcL) in control (C, 18 μM Mn) and *Arabidopsis* plants treated with 500 and 1500 μM Mn. (B) The relative polypeptide abundance was quantified by densitometric analysis (area × intensity bands) of the immunoblots and the presented data were normalized to the relative abundance of PsaA, PsaB, D1, PsbO, Lhcb1, and PbcL in control *Arabidopsis* plants. Mean ± SE were calculated from three independent experiments. Different lowercase letters indicate significant differences between the Mn treatments ($P < 0.05$).

the potential effect(s) of excess Mn on the functional/structural integrity of PSI remains elusive. As far as is known, the results presented in this study are the first report of an *in vivo* assessment of high Mn concentrations on PSI photochemistry. In contrast to PSII photochemistry, *in vivo* measurements of the oxidation state of P700 ($P700^+$) (Klughammer and Schreiber, 1991; Ivanov *et al.*, 1998), the primary donor of PSI demonstrated that the relative amount of oxidizable P700 ($P700^+$) decreased by 30% in Mn-treated *Arabidopsis* plants at concentrations above 1000 μM (Fig. 4 and Table 5). The functional impairment of PSI photochemistry by excess Mn was accompanied by a significant reduction in the abundance of PSI reaction centre polypeptides (PsaA and PsaB, Fig. 5). This clearly indicates that the major target of Mn toxicity within the photosynthetic electron transport chain of *Arabidopsis* is PSI- rather than PSII-related components. The reduced amounts of PSI reaction centre polypeptides PsaA and PsaB would imply acceptor side limitations of the photosynthetic electron transport and this could explain the increased reduction state Q_A in Mn-stressed plants reported earlier (Kitao *et al.*, 1997b). Moreover, the decreased expression of another Fe-containing chloroplastic protein precursor, ferredoxin-1

serving as a terminal electron acceptor of the photosynthetic electron transport, observed in Mn-treated young rice leaves also supports Mn-induced limitations at the acceptor side of PSI (Führs *et al.*, 2010).

One of the major mechanisms considered for Mn toxicity involves the inhibition of other essential cations including Fe, thus suggesting that a Mn-induced Fe deficiency may play a key role in the physiological responses to excess Mn (Foy *et al.*, 1978; Foy, 1984; Kohno *et al.*, 1984). More recently, chloroplast alterations in maize plants exposed to excess Mn (Doncheva *et al.*, 2009) and Mn toxicity in young rice leaves (Führs *et al.*, 2010) have been also ascribed to Mn-induced Fe deficiency rather than to direct Mn-induced oxidative stress. Given that about 80% of the plant Fe is located in the chloroplast (Terry and Abadia, 1986) and that the functional photosynthetic apparatus requires 22–23 iron atoms, of which PSI is the most Fe-abundant component (Ferreira and Straus, 1994), it seems reasonable to assume that the observed lower abundance of PSI reaction centre polypeptides and the associated decline of PSI photochemistry in Mn-treated *Arabidopsis* plants were consequences of a Mn-induced moderate Fe deficiency.

Although light energy is important for photosynthetic processes in plants, an excess of light can be also harmful because it can result in photoinhibition, which can be exacerbated when it is combined with other stresses (Powles, 1984; Aro *et al.*, 1993; Sonoike, 1996). Photoinhibition is a complex phenomenon that may cause damage to the photosynthetic apparatus reducing the photosynthetic efficiency when light conditions exceed the photon requirements for photosynthesis (Murata *et al.*, 2007). It is considered that PSII is the main site of photoinhibition (Aro *et al.*, 1993; Sonoike, 1996; Takahashi and Murata, 2008), being more unstable than PSI, because the D1 protein, one of the two major heterodimeric polypeptides of the PSII reaction centre complex, has a very high light-dependent turnover rate (Aro *et al.*, 1993; Burnap, 2004; Scheller and Haldrup, 2005; Takahashi and Murata, 2008). Earlier reports have shown that the susceptibility to Mn toxicity is strongly dependent on the light intensity and exposure of Mn-treated plants to high light can exacerbate the toxic effect of Mn (Horiguchi, 1988; Nable *et al.*, 1988; González *et al.*, 1998; Clair and Lynch, 2004; Hajiboland and Hasani, 2007). The present results also demonstrate that *Arabidopsis* plants predisposed to high Mn concentrations are more susceptible to photoinhibitory damage of PSII photochemistry in a concentration-dependent manner (Table 4).

Apart from the radiation-less dissipation of excess excitation energy in the chlorophyll pigment bed of LHCII, associated with the formation of the xanthophylls pigment zeaxanthin, which is considered one of the major protective mechanisms against photoinhibitory damage (Horton *et al.*, 1996; Niyogi, 1999), PSI-dependent cyclic electron transport has been also suggested to play a significant role in preventing the photoinhibitory damage of the photosynthetic apparatus during exposure of plants to high light conditions (Munekage *et al.*, 2002; Takahashi *et al.*, 2009). Considering the increased re-reduction rate of $P700^+$ in *Arabidopsis* (Table 5), the higher susceptibility of plants exposed to excess Mn to photoinhibition (Table 4) might be due to lower capacity of PSI-driven cyclic electron flow under conditions of Mn toxicity.

In summary, the results presented in this research demonstrate for the first time that exposure of *Arabidopsis* plants to excess Mn causes specific negative effects on the abundance of polypeptides comprising the reaction centre of PSI, thus resulting in decreased PSI photochemistry and lower capacity for cyclic electron transport, which may be due to a Mn-induced Fe deficiency and may have critical physiological implications under conditions of Mn toxicity in higher plants.

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