Journal of Plant Physiology 183 (2015) 138–143

Contents lists available at ScienceDirect

Journal of Plant Physiology

journal homepage: www.elsevier.com/locate/jplph

Short communication

Zinc deficiency affects physiological and anatomical characteristics in maize leaves

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ARTICLE INFO

Article history:
Received 10 March 2015
Received in revised form 27 May 2015
Accepted 27 May 2015
Available online 12 June 2015

Keywords:
Corn
Nutritional diagnostic
Photosynthesis

ABSTRACT

Zinc (Zn) is an essential microelement involved in several plant physiological processes. Therefore, it is important to identify Zn deficiencies promptly—before extensive damage occurs to the plant. The diagnostic tools that are used to identify Zn deficiencies are very important in areas where Zn deficiencies occur. Such diagnostic tools are vital for nutritional management and fertilizer recommendations. The current study investigated the effects of Zn deficiency on maize plants by recording a number of physiological and anatomical parameters. A Zn omission trial (from 0 to 22 days) was carried out to produce plants that had varying degrees of Zn deficiency. Typical symptoms of Zn deficiency (e.g. chlorotic stripes and purple shades on the edges and leaf sheath) appeared 16 days after the omission of Zn from nutrient solutions. As the time of Zn omission increased, there were significant decreases in net photosynthesis, stomatal conductance, maximal efficiency of photosystem I (evaluated by F_v/F_m), biomass (dry weight) and Zn concentrations in plants. Zinc-deficient plants also had a lower vascular bundle proportion coupled with a higher stomata density. These physiological and anatomical changes negatively impacted plant growth. Moreover, they occurred before visible symptoms of Zn deficiency were observed. Zinc concentrations were recorded for younger leaves, rather than for more mature leaves, which is usually recommended for plant analysis. The results demonstrate that the analysis of Zn in young leaves of maize is a very sensitive indicator of Zn status.

Introduction

Zinc (Zn) deficiency is the most widespread micronutrient deficiency problem globally. It is commonly associated with reductions in crop yield (Cakmak et al., 1996; Wissuwa et al., 2006; Hossain et al., 2008; Hafeez, 2013; Mousavi et al., 2013) and food quality (Cakmak, 2008; Cakmak et al., 2010a; Velu et al., 2014). Therefore, when Zn fertilization is used, crop productivity usually increases. This has been demonstrated for a variety of crops, in particular for maize (*Zea mays* L.) (Bukvic et al., 2003; Potarzycki and Grzebisz, 2009; Galavi et al., 2011; Wang et al., 2012).

The function of Zn in plants has been extensively studied (Cakmak, 2000; Wang and Jin, 2005; Sharma et al., 2013; Höller et al., 2014). Zinc plays a key role as a structural constituent and regulatory co-factor in a wide range of enzymes and proteins (Broadley et al., 2007; Hänsch and Mendel, 2009; Figueiredo et al., 2012). The vast majority of plant enzymes that are activated by Zn are involved in carbohydrate metabolism, maintenance of cell membrane integrity, protein synthesis and in the regulation of auxin synthesis(Skoog, 1940; Coleman, 1992). It should be noted that when plants are deficient in Zn, protein synthesis is reduced as amino acids and amides accumulate in plant tissues (Marschner, 1995). Zinc is also essential for tryptophan biosynthesis, which is fundamental for auxin formation(Mašev and Kutáček, 1966; Marschner, 1995). It has been observed that under some specific conditions, leaf concentrations of tryptophan may increase due to the impairment of protein and auxin synthesis (Marschner, 1995). The importance of Zn to enzyme function is particularly evident in chloroplasts and the cytoplasm. In these organelles, several enzymes are dependent on Zn for photosynthesis, biomass production and for the prevention of cell membrane damage (Cakmak, 2000).

The diagnosis of Zn plant status is important to ensure optimal crop productivity and food quality. Currently, there are several methods that can be used to diagnose plant nutritional status. However, the two most commonly used methods are (1) examination

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http://dx.doi.org/10.1016/j.jplph.2015.05.014
0176-1617/

of visual symptoms and (2) analysis of plant Zn concentrations. Zinc deficiency symptoms initially appear on young leaves and meristems of plants due to the low mobility of Zn. Such symptoms include leaf chlorosis and a decrease in leaf size, which in turn causes stunting and a decrease in the number of tillers (Cakmak et al., 1998; Mousavi, 2011). It is important to note that visual symptoms of Zn deficiency normally occur when plants are suffering severe stress. Therefore, using visual inspection as a method for diagnosing Zn deficiency is considered a late diagnostic tool. In this case, it is reasonable to assume that late diagnosis reduce the possibility to correct the problem by fertilization—particularly for annual crops. However, the information obtained can still be of crucial importance for the next crop and for perennial crops. Before the appearance of visible symptoms, other changes such as those to physiological, anatomical and chemical parameters may occur. Monitoring such modifications are important in order to understand and manage plant nutrition.

Although several studies have investigated nutritional status and the anti-oxidative responses of Zn deficiency in maize (Singh et al., 2005; Wang et al., 2009; Afsharnia et al., 2013; Hafeez, 2013) and other plants (Cakmak et al., 1997, 1998; Daneshbakhsh et al., 2012, 2013; Impa and Johnson-Beebout, 2012; Höller et al., 2014), the physiological and anatomical responses of maize under Zn deficiency is largely unknown. In addition, it is still debated whether the chemical analysis of young leaves is suitable for diagnosing Zn deficiencies in maize. The two aims of this study were to (1) investigate the effects of Zn deficiency on physiological and anatomical parameters as well as the chemical composition of the leaf and (2) to determine if the analysis of young leaves could be used as a diagnostic tool for Zn status in maize.

Materials and methods

Plant and experimental design

The experiments were carried out in Viçosa (20°45′S, 42°54′W, 650 m altitude) in south-eastern Brazil, during January and February 2003. Maize (*Zea mays* L.) plants from hybrid BRS1010 were hydroponically grown in plastic pots (1.5 L volume) in a greenhouse under semi-controlled conditions. The greenhouse temperature was maintained at 30 ± 5 °C and had a light intensity of 1500 μmol photons m^{-2} s^{-1} (maximum photosynthetic photon flux density, PPFD).

After germination in Germitest® paper, seedlings were transferred to plastic pots. Zinc was supplied as $ZnSO_4$ at concentrations of 0 μmol L^{-1} (for the control treatment) and 2.4 μmol L^{-1} de Zn (for Zn-sufficient plants). Further details of methodologies including composition of the nutrient solutions and the control of pH have been described elsewhere (Ruiz, 1997).

Nutrient solutions were prepared with distilled water that had an electric conductivity <3 μS m^{-1}. Chemical analysis of nutrient solutions showed that Zn contamination was ≤0.1 μmol L^{-1}, which can be considered very low for plant growth. The experiment had a completely randomized design, with four plants in individual pots per treatment combination, i.e. four replicates per treatment. Sampling and measurements were performed at 0, 2, 6, 10, 14, 18 and 22 days (d) after Zn omission. The pots were randomized periodically to minimize any variation amongst treatments.

Physiological parameters

The leaf gas exchange parameters and measurements of chlorophyll (Chl) *a* fluorescence were determined 22 days after Zn omission. Chl-*a* fluorescence (Chl fluorescence) parameters were measured before harvest using a portable fluorometer (Plant Efficiency Analyser-PEA, Hansatech, King's Lynn, UK). All measurements were performed on young leaves (second leaf on the last stem with visible ligule). Leaves were acclimated in the dark for 20 min before measurements were taken. The time of measuring was 5 s, and irradiance was set at 75% of the maximum (>3000 μmol m^{-2} s^{-1}). Initial (F_0), maximum (F_m), variable ($F_v = F_m - F_0$), F_v/F_0, and F_v/F_m were recorded. F_v/F_m was used to calculate the potential maximum quantum yield of PSII, F_v/F_0 was used to assess PSII activity. The area under the fluorescence curve between F_0 and F_m was also calculated. The ratio of this area to the value of F_m–F_0 was used to estimate the relative pool size of plastoquinone (PQ).

Gas exchange parameters were determined by using a cross-calibrated portable open-flow gas exchange systems (LI-6400XT, LI-COR, Lincoln, NE, USA). The net CO_2 assimilation rate (A) and stomatal conductance to water vapor (g_s) were measured on attached, fully expanded leaves from 8:00 to 11:00 h (solar time), which is when A was at its maximum, under artificial PPFD, i.e. 1,000 μmol m^{-2} s^{-1} at the leaf level and 400 μmol CO_2 mol^{-1} air^{-1}. All measurements were performed by fixing the block temperature at 25 °C, maintaining the vapor pressure deficit at approximately 1.0 kPa and setting the amount of blue light to 10% of the PPFD to optimize the stomatal aperture.

Anatomical parameters

At the end of the experiment (22 days), leaf samples (2 cm^2) were collected from the mid region of the same leaf used for physiological measurements. The material was fixed in formaldehyde, acetic acid and 50% ethanol (FAA50) at 5:5:90 (v:v:v) for 48 h with adaptations from (Johansen, 1940) and stored in 70% ethanol. After fixation, an area of the leaf (approximately 25 mm^2), in the midline portion, was transferred into a series of ethanol solutions (from 50 to 100% (v v^{-1})) at increasing ratios for dehydration. The samples were then embedded transversally using an Leica Historesin Embedding Kit (Heidelberg, German), according to the manufacturer's recommendations.

Transversal sections of 8 μm thickness were taken from the embedded tissues with a rotary microtome with automatic advance (RM 2155, Leica, Deerfield, IL, USA) and transferred onto slides from the distilled water bath and dried.

The tissues on the slides were stained with toluidin blue pH 4 (O'Brien et al., 1964) and coverslipped using a mounting medium (Permount™).

Digitalized images were obtained with a light microscope (Model AX70TRF, Olympus optical, Tokyo, Japan) that was connected to a digital camera (U-photo system) and microcomputer. The area and count data were measured using the software Image Pro® Plus version 4.1 (Media Cybernetics, Inc., Silver Spring, USA). The perceptual area for each tissue was calculated in relation to the total leaf cross-sections area.

For stomata density evaluation, an epidermis impression was collected using the instant glue method, that has been described previously (Rodella et al., 1992), on both sides of the leaf. Stomata count was performed using a light chamber attached to a light microscope in five randomly chosen microscopic fields by repetition and then converted to the number of stomata per mm^2.

Harvest and chemical analysis

Plants were harvested at V7 vegetative growth stage, and divided into shoots and roots. These plant tissues were oven-dried at 70 °C until a constant weight was reached. The same leaves that were used for physiological and anatomical analyses were dried and digested in HNO_3–$HClO_4$ and the extract was analyzed for Zn by Inductively Coupled Plasma—Atomic Emission Spectrometry ICP-AES (Perkin-Elmer, model Optima 3300 DV).

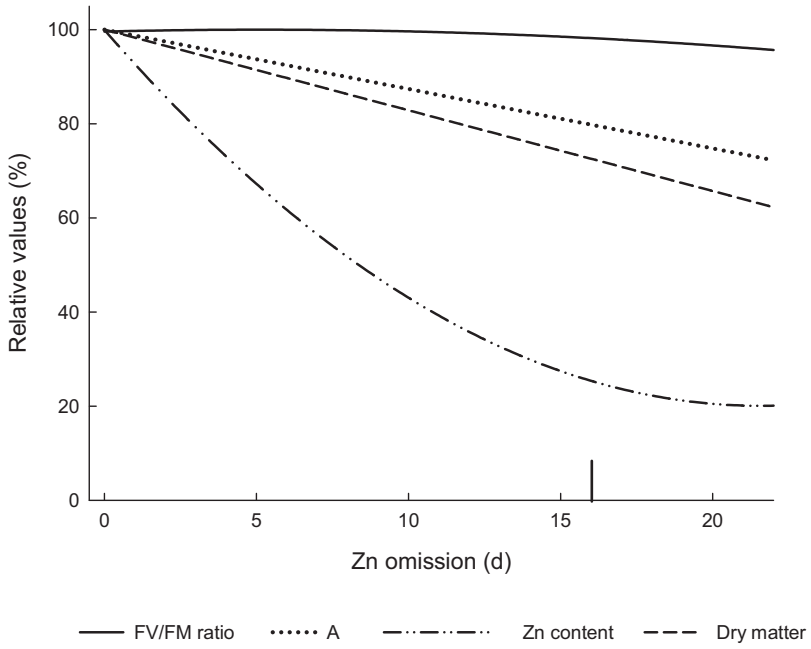

Fig. 1. Relative values of F_v/F_m ratio, leaf CO_2 assimilation rate (A), dry matter and concentration of Zn in maize plants with Zn omission in nutrient solution. The vertical dash (|) indicates the beginning of visual symptoms of Zn deficiency.

Statistical analysis

Data was statistically analyzed using ANOVA evaluating effects of time of Zn omission in nutrient solution on F_v/F_m ratio, A, dry matter, Zn leaf concentration by means of regression. Data was converted to relative values so that all parameters could be compared on the same graph. Relative values were calculated as a percentage of the control response (i.e. prior Zn omission). Anatomical data were compared by Least Significant Difference (LSD): $p < 0.05$

Results

Plant growth and physiological parameters

After 16 days of Zn omission, visual Zn deficiency symptoms were apparent; including chlorotic stripes and purple shades on leaf edges and the leaf sheath. Following Zn omission, the F_v/F_m ratio and A decreased (X and Y%, respectively), which was followed by significant reductions in the amount of dry matter and leaf Zn concentrations (Table 1 and Fig. 1). Accordingly, when plants first exhibited visible symptoms, a number of other parameters were also reduced; Zn leaf concentrations, dry matter, photosynthesis and F_v/F_m were reduced by 75 28, 20 and1.2%, respectively, compared with Zn-sufficient plants.

Maize plants growing with no Zn limitation had an estimated foliar concentration of Zn of 39.6 mg kg^{-1} whereas in plants that had visible symptoms, the concentration was <10 mg kg^{-1}.

Regardless of the time of Zn omission, Zn deficiency caused A and g_s to both decreases (Fig. 2). A was reduced by approximately 25% in Zn-deficient plants after 22 days of Zn omission (Table 1). For Zn-sufficient plants, the average rate of A was 32 μmol m^{-2} s^{-1} and for Zn-deficient plants (22 days of Zn omission) it was 23 μmol m^{-2} s^{-1}.

Zn deficiency notably decreased both g_s and E in maize plants following 6 days omission in nutrient solution (Fig. 2). A correlated logarithmically with g_s ($r^2 = 0.90$, $p < 0.001$) during Zn omission in maize plants. This suggests that stomatal limitations were

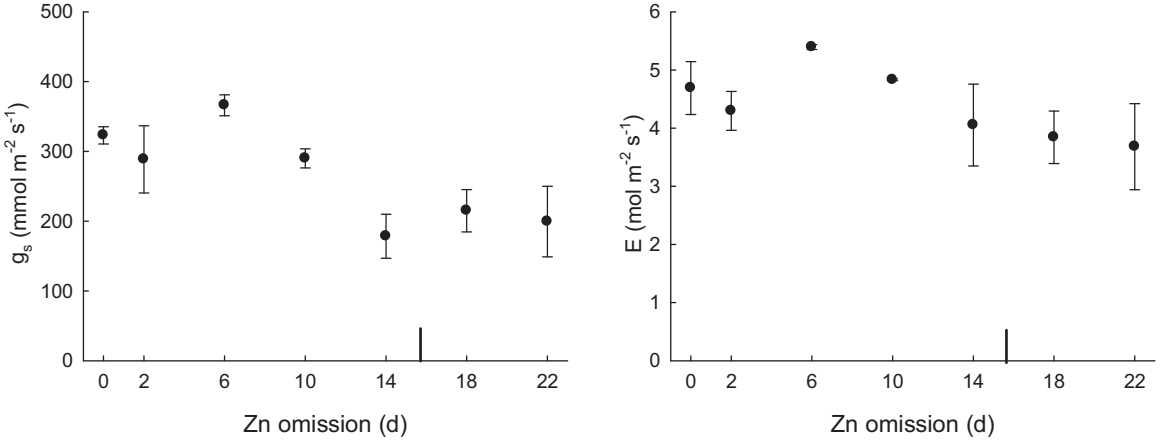

Fig. 2. Stomatal conductance (g_s) and transpiration (E) in maize plants with Zn omission in nutrient solution. The vertical dash (|) indicates the beginning of visual symptoms of Zn deficiency.

Table 1
Zn deficiency alters growth and physiological parameters in maize plants. Mean values and regression equation of F_v/F_m, CO_2 assimilation rate (A), dry matter and Zn foliar concentration following Zn omission in nutrient solution are presented.

Variable	Time of Zn omission (d)						
	0	2	6	10	14	18	22
F_v/F_m ratio	0.803	0.802	0.799	0.805	0.797	0.781	0.770
A (μmol m^{-2} s^{-1})	31.30	30.64	31.04	29.09	25.55	24.69	22.93
Dry matter (g plant^{-1})	4.47	5.71	5.10	4.22	3.92	3.86	3.04
Zn concentration (mg kg^{-1})	40.33	33.99	22.01	19.07	11.48	9.20	7.60
Regression equation[a]							
F_v/F_m ratio		$\hat{y} = 0.80 + 0.001x - 0.0001x^2$			$R^2 = 0{,}942$		
A (μmol m^{-2} s^{-1})		$\hat{y} = 32.05 - 0{,}4045x$			$R^2 = 0{,}931$		
Dry matter (g plant^{-1})		$\hat{y} = 5.125 - 0,090x$			$R^2 = 0{,}716$		
Zn concentration (mg kg^{-1})		$\hat{y} = 39.605 - 2.938x + 0.0681x^2$			$R^2 = 0{,}948$		

[a] All coefficients are significant by t test ($p < 0.05$).

Table 2
Mean values of anatomical characteristics of transversal section of laminar blade of maize with Zn omission in nutrient solution.

Variable	Time of Zn omission (d)					LSD
	0	6	14	22	average	
Total area (μm^2)	238 790	229 337	210 669	216 035	223707	57 595
Mesophyll area (%)	45	43	45	45	44	5.2
Epidermis area on the adaxial (%)	14.38	15.34	14.34	16.22	15.07	2.9
Epidermis area on the abaxial (%)	10.70a	10.79a	9.06b	11.01a	–	1.4
Bundle sheath cells (%)	12.15	11.37	11.97	12.04	11.88	1.9
Vascular bundle area (%)	8.69ab	8.81a	7.27ab	6.96b	–	1.8
Sclerenchyma area (%)	1.87	1.58	2.03	1.76	1.81	0.6
Ar space(%)	6.79	9.23	9.78	7.05	8.21	3.8
Distance betwem bundle (μm)	171	177	170	149	167	38
Leaf thickness (μm)	195	188	172	176	183	46
Stomata density (stomata mm^{-2})						
adaxial	34b	37ab	35b	41a	–	5.3
abaxial	56b	62ab	58ab	64a	–	7.2

LSD: Least Significant Difference ($p < 0.05$). Significant differences between treatments are indicated by different letters.

responsible for the reductions in *A*; reductions that are clearly associated with growth impairments and were observed in Zn-limited plants.

Leaf symptoms and anatomical parameters

To further understand the effects of Zn deficiency on maize plants, a range of visual and anatomical parameters were analyzed. Unsurprisingly, Zn-deficient plants were characterized by strong reductions in shoot elongation and leaf size. In addition, young leaves displayed chlorotic stripes adjacent to the midrib, and purple shades on the edges and leaf sheath. Compared with Zn-sufficient plants, the size of the vascular bundle was reduced in Zn-deficient plants. Stomata density was also higher in Zn-deficient plants (Table 2); most likely due to the reduction in leaf size. There were no significant differences between treatments for any of the other anatomical parameters that were evaluated (Table 2). In terms of the distribution of tissue types, the foliar blade comprised of 44% mesophyll, 8% intercellular space and 1.8% sclerenchyma. The epidermis area on the adaxial and abaxial surfaces corresponded to 15 and 10%, respectively, of the maize foliar blade. Stomata density ranged from 34 to 41 stomata per mm^2 on the adaxial surface and from 56 a 60 stomata per mm^2 on the abaxial surface.

Discussion

In recent years, the level of understanding in regards to plant responses to Zn deficiencies has increased, yet the effects on physiological and anatomical parameters is still unknown. Zinc deficiency has been a serious challenge in several parts of world as it decreases crop yields and food quality (Alloway, 2009; Cakmak et al., 2010b; Sadeghzadeh, 2013; Velu et al., 2014). Diagnosing Zn deficiency, by either visual field observations or plant analysis, has been efficiently and widely used; particularly in cases of severe deficiency. The current study demonstrates that before visual symptoms are observed, physiological damage can occur, which in turn, may decrease yield. The results demonstrate that Zn concentrations in young leaves can be used as a sensitive indicator of the Zn status in maize plants. In these plants, sufficient leaf Zn concentrations range from 20 to 70 mg kg^{-1} (Martinez et al., 1999). In the current study, Zn concentrations in Zn-sufficient plants were approximately 40 mg kg^{-1}, whereas in Zn-deficient plants, concentrations were <10 mg kg^{-1}. However, it should be noted that that younger leaves were used for Zn diagnostic tests. The official recommendation for plant analysis sampling usually focuses on mature leaves and therefore the interpretation of such results should be taken with caution for immobile nutrients (Jones et al., 1991; Martinez et al., 1999). Thus, it is tempting to suggest that Zn deficiency can be achieved, with a considerable homeostasis, in younger leaves than usually recommended. It should be stressed that the mobility of Zn in plants is restricted and varies between plant species and genotypes (Wolff et al., 2013). Therefore, using mature leaves or not a specific leaf to diagnose Zn deficiency may be an unreliable approach (Brennan et al., 1993). Moreover, the total concentration of Zn in plant tissues has often been considered an unsatisfactory parameter for evaluating Zn nutritional status (Cakmak et al., 1997, 1998; Wissuwa et al., 2006). To overcome this, previous studies have suggested that the activity of Cu/Zn-superoxide dismutase (SOD) is a better indicator of Zn nutritional status than the Zn concentration itself (Cakmak et al.,

1997). Nevertheless, further investigation of the reliability of this method in other important crops should be a priority for future studies.

Following Zn restriction, the photosynthetic capacity of maize plants was clearly reduced. This reduction was most likely due to: (1) the decrease in intercellular CO_2 concentration (C_i) (Supplementary Material), which may have led to biochemical limitations to *A;* (2) stomatal limitations may have caused *A* to decrease; and, (3) changes to the capacity of photosystem II, which has been shown in this study, and by others (Wang and Jin, 2005; Hajiboland and Amirazad, 2010), to reduce *A* via the generation of reactive oxygen species, which may interfere in photosynthetic metabolism. Chl fluorescence kinetics showed that the maximum quantum efficiency of PSII has a tendency to decrease in Zn-deficient plants. Accordingly, it has been demonstrated previously that reductions in F_v/F_m are usually accompanied by an enhanced production of superoxide (Cakmak and Marschner, 1993; Cakmak, 2000). However, others factors that are associated with Zn deficiency have also been described, such as the decrease in carbonic anhydrase activity (Hacisalihoglu et al., 2003; Wolff et al., 2013), the accumulation of saccharides in leaves (Cakmak, 2000; Marschner, 1995) and oxidative stress (Cakmak and Marschner, 1993; Sharma et al., 2004; Höller et al., 2014). Combined with these past studies, the current results suggest that the induction of anti-oxidative responses during Zn deficiency may occur before visual symptoms are apparent, and that oxidative stress can be also an early sign of Zn deficiency in plants. Therefore, a priority for future studies would be to investigate the anti-oxidative mechanism in plants. This would further our understanding of the key responses following Zn deficiency (Cakmak and Marschner, 1988; Höller et al., 2014).

Zinc deficiency most likely impaired plant growth because of the direct effects of Zn on gas exchange and Chl-a fluorescence parameters (Table 1 and Fig. 1). In plants with visible Zn deficiency symptoms, *A* was reduced by 20%. This is in good agreement with the assumption that Zn deficiency decreases net photosynthesis by 50−70% (depending on the plant variety and the severity of Zn deficiency) (Wang and Jin, 2005; Afsharnia et al., 2013). Although it is well established that plants must achieve a balance between carbon assimilation, carbon storage and growth, remarkably little is known about the importance of these linkages for sustained plant growth in response to Zn deficiency. Despite the fact that we have provide circumstantial evidence for an important role of Zn status in governing those parameters, we cannot rule out that the Zn effects on the studied parameters could have been influenced by other nutrients, particularly macronutrients. However, the close linkage between Zn deficiency and N and S uptake and concentration in land plants which is based in the key role of those nutrients in protein synthesis, has been already reported (Marschner, 1995). It has also been demonstrated that increases in Zn concentration in crops are positively correlated with N and S absorption (Hossain et al., 2008). While the exact mechanism by which these nutrients are connected seems to be unclear from our study, it clearly remains as important points that should be addressed in future research.

Zinc deficiency affected leaf elongation and increased stomata density. This response was associated not only with decreased plant growth but also with anatomical and physiological changes. Interestingly, the anatomical changes observed here demonstrated that in Zn-deficient plants, the absence of a tight packing around the vascular bundles is likely associate with a limited exposure to intercellular air spaces. The results obtained here also suggest that the reduction in vascular bundle size, as a result of Zn deficiency, can affect the transport of water and nutrients from roots to shoots. This effect may be associated with limitation to both transpiration efflux and phloem loading. Accordingly, the combined effects of Zn deficiency and drought stress have been related to cause chloroplast damage, increased production of reactive oxygen species (ROS) and reduction of photosynthesis following stomatal limitations (Disante et al., 2010; Hajiboland and Amirazad, 2010b; Kasim, 2007). Increasing our understanding of how plants sense and adapt to variations in Zn availability, will contribute to the development of improved crops; specifically, crops that are more efficient at acquiring and using Zn. Given that in Zn-sufficient plants, the negative impacts of drought seems to be alleviated (Disante et al., 2010; Upadhyaya et al., 2013), this would not only help to overcome yield problems but also mineral and water deficiency in places subjected to drought episodes as well as with low bioavailability of Zn in soils (Assunção et al., 2010).

Given the relative importance of Zn nutrition in maize, the results presented here provide evidence for interconnected networks that control plant growth under Zn limited conditions. This study also verifies previous research that has shown that maize is sensitive to Zn deficiency and that stunted growth and chlorotic leaves are symptomatic of plants under Zn deficiency. Most importantly, the results show that before visible symptoms of Zn deficiency are apparent, a number of physiological and anatomical parameters are first affected. These changes to plant parameters negatively affect plant growth. Overall, our results demonstrate that the analysis of Zn concentrations in younger leaves, rather than in more mature leaves, can be effectively used as a very sensitive indicator of the Zn status in the plant.

Acknowledgements

This work was supported by funding from Coordination for Scientific Support for Post-Graduate Level Training-CAPES, Brazil. We also acknowledge Dr Casey Doolette (The University of Adelaide) for critical review of and suggestions regarding this work.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jplph.2015.05.014

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