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BRIEF COMMUNICATION

Photosynthetic and anatomic responses of peanut leaves to zinc stress

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Abstract

In this study, photosynthetic performance, pigment content, chlorophyll *a* fluorescence, and leaf anatomy in peanut (*Arachis hypogaea*) subjected to zinc (Zn) stress were investigated. Zn stress resulted in reduction of photosynthetic and transpiration rates, pigment contents and root biomass. Zn-induced xerophyte structure in peanut leaves (*i.e.* thick lamina, upper epidermis, and palisade mesophyll, as well as abundant and small stomata) also contributed to decreased transpiration rate and stomatal conductance. This in turn, partially contributed to the limitation of photosynthesis.

Additional key words: *Arachis hypogaea*, chlorophyll *a* fluorescence, gas exchange, leaf anatomy

Although zinc is an essential element for normal plant growth, it is highly phytotoxic at high concentrations. In most crops, the required Zn concentration in a plant leaf for adequate growth is about 15 - 20 mg(Zn) kg^{-1}(d.m.) and toxicity symptoms usually become visible at 300 mg(Zn) kg^{-1}(leaf d.m.). However, some crops may show toxicity symptoms at 100 mg(Zn) kg^{-1}(d.m.) (Marschner 1995, Broadley *et al*. 2007). Likewise, toxicity thresholds can be highly variable even within the same species (Broadley *et al.* 2007). The most significant phytotoxicity symptoms of Zn were growth stunting, biomass reduction, Fe-deficiency-induced chlorosis owing to reduction in chlorophyll (Chl) synthesis and chloroplast degradation, and interference with uptake of P, Mg, and Mn (Rout and Das 2003, Broadley *et al.* 2007).

The negative effect of excess Zn on photosynthesis has been known for a long time. Despite the well-established negative effects of Zn on chlorophyll content (Macfarlane and Burchett 2001, Monnet *et al.* 2001, Ralph and Burchet 1998), enzyme activities (Chaney 1993, Monnet *et al.* 2001, Vaillant *et al*. 2005, Van Assche and Clijsters 1986b), and photochemical processes (Monnet *et al*. 2001, Ralph and Burchet 1998, Vaillant *et al.* 2005, Van Assche and Clijsters 1986a), a clear picture of overall photosynthetic response to excess of Zn has yet to be fully appreciated.

Several reports have demonstrated that excess of heavy metals (Cd, Pb, Ni, and Mn) induced changes in leaf anatomy (Kovačević *et al.* 1999, Papadakis *et al.* 2007). However, little is known about the responses of leaf structure to Zn stress. There is also an insufficient knowledge on the correlation between leaf anatomic traits and CO_2 assimilation under heavy metal stress. Therefore, in this study, we investigated the effects of Zn stress on photosynthesis and anatomy of peanut leaves.

Seedlings of peanut (*Arachis hypogaea* cv. Luhua 11, 14-d-old) were grown in a Hoagland nutrient solution (pH 6.5) containing 0.2 (control), 200, 500, or 1000 μM $ZnSO_4 \cdot 7H_2O$, in a chamber with a 16-h photoperiod (irradiance of 500 μmol m^{-2} s^{-1}), day/night temperatures 23/18 ± 2 °C and relative humidity 60 ± 5 %. Each treatment had three replicates (two uniform plants per replicate).

After 21 d of Zn exposure, the plants were harvested, and the roots were soaked in 20 mM Na_2-EDTA for 15 min to remove Zn adhering to root surfaces. The roots and shoots were separated and oven-dried at 105 ºC for 30 min and then at 70 °C until they reached constant masses.

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Abbreviations: Car - carotenoids; Chl - chlorophyll; c_i - intercellular CO_2 concentration; E- transpiration rate; F_0 - minimal fluorescence; F_m - the maximal fluorescence; F_v - variable fluorescence; g_s - stomatal conductance; PS 2 - photosystem 2; P_N - net photosynthetic rate; WUE - water use efficiency; Φ_{PS2} - the effective quantum yield of PS 2.

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The dried tissues were weighed and ground into powder for the determination of Zn content. This was measured by flame atomic absorbance spectrometry after it was digested with mixed acid [HNO_3 + $HClO_4$ (3:1, v/v)].

The net photosynthetic rate (P_N), transpiration rate (E), stomatal conductance (g_s) and intercellular CO_2 concentration (c_i) were determined with a portable photosynthesis system (*LiCor-6400*; Lincoln, NE, USA). This experiment was conducted at irradiance of 500 μmol m^{-2} s^{-1}, leaf temperature of 25 °C, and CO_2 concentration of 380 ± 5 μmol(CO_2) mol^{-1}. From these data, the water use efficiency was estimated (WUE = P_N /E).

Mature leaves (0.2 g) were extracted in the dark at 4 ºC with 5 cm^{-3} mixture of acetone and ethanol (1:1), absorbance at 663, 645 and 470 nm was measured by spectrophotometer (*UV-2802*, Unico, USA) and Chl and carotenoid (Car) contents were calculated according to Lichtenthaler (1987). The Chl *a* fluorescence parameters were measured using the *Mini PAM* (*Walz*, Effeltrich, Germany) after a 1-s pulse of red light on dark-adapted (30 min) leaves, the minimum fluorescence (F_0), the maximum fluorescence (F_m), the variable to maximal fluorescence ratio (F_v/F_m), and the variable to minimal fluorescence ratio (F_v/F_0) values were determined. Furthermore, the effective quantum yield of PS 2 ($\phi_{PS\ 2}$) was determined by applying a saturating light pulse (5000 μmol m^{-2} s^{-1}; 1 s) under ambient irradiance (500 μmol m^{-2} s^{-1}).

The sections (5 × 10 mm) were excised from the middle of the lamina, along with the mid-rib, and fixed in FAA (formaldehyde : acetic acid : 50 % ethanol, 5:5:90). The samples were dehydrated in an increasing alcohol concentration, embedded in paraffin, sectioned using an ultra microtome (12 μm thick) and stained with fast green. The stomatal density and length were determined for the adaxial and abaxial surfaces of each leaf using prints made with nail varnish. All anatomical characteristics were measured using the *PHMIAS 2003* software with a *ME200* photomicroscope.

The analysis of variance (*ANOVA*) for all the measured variables was performed by *SPSS* ver. *11.5* software, and differences between means were determined using the Duncan's multiple range test.

Zn accumulation in the roots and shoots increased dramatically with increasing Zn concentration in solution (Table 1). Zn content in roots of Zn treatments were about 2.6- to 2.9-fold higher than that in the shoots. Moreover, no significant differences were found in the shoot Zn content between the 500 and 1000 μM Zn treatments. Increase of Zn concentration caused obvious retardations of root growth; nonetheless, it did not affect the shoot biomass (Table 1).

The results showed that the elevated Zn caused a decrease in the P_N in the peanut leaves. This was accompanied with a decrease of E and g_s, as well as an increase of c_i (Table 1). Khudsar *et al*. (2004) observed similar results in *Artemisia annua*. The parallel change of P_N and g_s in peanut leaves reinforce evidence that the changes in P_N could be mainly attributed to the changes in g_s (Jones 1992, Chartzoulakisa *et al*. 2002).

In the present study, the F_v/F_m and F_v/F_0 decreased with the increasing of Zn concentration, whereas $\phi_{PS\ 2}$ remained unchanged except in the 500 μM Zn treatment (Table 1). The decrease in the F_v/F_0 ratio reflects the changes in the thylakoid structure on the donor site of the PS 2, whereas, the reduction in F_v/F_m indicates decrease in efficiency of the PS 2 (Skorzynska-Polit and Baszynski 1997). No significant correlation was observed between Chl fluorescence and gas exchange parameters indicating that the stomatal limitation to photosynthesis might be predominant.

Heavy metals have been found to decrease the Chl content and the Chl *a*/*b* ratio, and increase the Chl/Car ratio in many terrestrial plants (Ouzounidou 1993, Moustakas *et al*. 1994, Khudsar *et al*. 2004). In peanut, Chl *a*, Chl *b* and Car contents slightly decreased under Zn stress in comparison with control but they were not further affected by increasing Zn concentration. These parameters were correlated with P_N (Table 1). The results suggest that P_N inhibition could be, at least partially, caused by Chl content reduction. The unaltered Chl *a*/*b* ratio indicated stability in the PS 2/PS 1 ratio (Váradi *et al.* 2003) and/or in the antennae composition (Durnford *et al.* 2003). Similar results were described by Monnet *et al.* (2001). The higher Car/Chl ratio under 200 μM Zn stress suggested a defense potential of leaves against photoinhibition (Behera *et al*. 2002).

We found that Zn treatments result in an increase in the thickness of lamina, upper epidermis, and palisade, as well as induction of small but abundant stomata (Table 1). These traits were usually considered as xerophytic characteristics of leaves (Bosabalidis and Kofidis 2002, Sundberg 1986, Fahn 1964). It might be a response to physiological drought caused by Zn *via* a decrease in the root growth. In this study, we focused on the changes in leaf anatomy because they play important roles for CO_2 and water diffusion (Evans *et al*. 1994, Syvertsen *et al.* 1995). The positive correlations of P_N, E, and g_s with the stomatal length in the upper epidermis and the lower epidermis thickness, as well as the negative correlation of P_N with the lamina thickness and palisade tissue thickness were observed. Small and abundant stomata might maximize the rate of carbon gain while minimizing water loss (Bosabalidis and Kofidis 2002). Thicker palisade tissue increased the CO_2 absorbing surface of the mesophyll (Rhizopoulou and Psaras 2003). The mesophyll compartmentalization protected the leaf against water stress (Terashima 1992). However, it unavoidably increased the CO_2 diffusion pathway in the tissue (Miyazawa and Terashima 2001).

Stomatal limitation is often considered as an early physiological response to water deficit, which results in decreased P_N, through limited CO_2 availability in the mesophyll (Cornic 2000). However, there is strong evidence that photosynthetic processes in the mesophyll such as Rubisco activity, RuBP regeneration, ATP supply, electron transport rate, and light capture efficiency in the

Table 1. The effect of different Zn concentrations on growth, gas exchange, chlorophyll fluorescence, photosynthetic pigments, and anatomic traits of leaves in *A. hypogaea*. Means ± SE, n =3 for Zn content, biomass and pigment content, 18 for gas exchange and Chl fluorescence parameters, 60 for stomatal characters and 36 for other anatomic traits.. Means in the same row followed by the same letters are not significantly different at $P < 0.05$ based on Duncan's multiple range test.

	0.2 μM	200 μM	500 μM	1000 μM
Root Zn content [mg g^{-1}(d.m.)]	0.13 ± 0.00d	2.74 ± 0.28c	3.47 ±0.55b	3.81 ± 0.88a
Shoot Zn content [mg g^{-1}(d.m.)]	0.07 ± 0.00c	1.05 ± 0.12b	1.25 ±0.23a	1.29 ± 0.16a
Root biomass [g $plant^{-1}$]	0.51 ± 0.01a	0.43 ±0.01b	0.27 ± 0.01c	0.26 ± 0.03c
Shoot biomass [g $plant^{-1}$]	1.55 ± 0.06a	1.50 ±0.07a	1.45 ± 0.05a	1.38 ± 0.03a
P_N [μmol (CO_2) m^{-2} s^{-1}]	8.4 ± 0.4a	5.7 ± 0.3b	5.0 ± 0.2c	4.7 ± 0.2c
g_s [mol (H_2O) m^{-1} s^{-1}]	0.03 ± 0.00a	0.02 ± 0.00b	0.01 ± 0.00c	0.01 ± 0.00c
E [mmol (H_2O) m^{-2} s^{-1}]	4.8 ± 0.3a	2.7 ± 0.4b	1.6 ± 0.1c	1.7 ± 0.1c
c_i[μmol (CO_2) mol^{-1}]	187 ± 4c	258 ± 8b	291 ± 12a	313 ± 11a
WUE	1.8 ± 0.1c	2.6 ± 0.2b	3.3 ± 0.2a	3.0 ± 0.2ab
F_v/F_m	0.82 ± 0.00a	0.81 ± 0.01ab	0.81 ± 0.00ab	0.80 ± 0.00b
F_0/F_m	4.6 ± 0.1a	4.3 ± 0.2ab	4.2 ± 0.1ab	4.0 ± 0.1b
ϕ_{PS2}	0.76 ± 0.00a	0.74 ± 0.01a	0.69 ± 0.025b	0.72 ± 0.01ab
Chl a [mg g^{-1}(d.m.)]	36.4 ± 2.4a	25.2 ± 2.6b	24.9 ± 1.4b	22.8 ± 1.6b
Chl b [mg g^{-1}(d.m.)]	8.9 ± 1.0a	6.1 ± 0.7b	6.0 ± 0.6b	5.5 ± 0.5b
Chl [mg g^{-1}(d.m.)]	45.3 ± 3.5a	31.3 ± 3.3b	30.9 ± 1.9b	28.3 ± 2.1b
Car [mg g^{-1}(d.m.)]	7.2 ± 0.6a	4.5 ± 0.6b	4.8 ± 0.4b	4.4 ± 0.4b
Chl a/b	4.12 ± 0.21a	4.13 ± 0.08a	4.18 ± 0.20a	4.20 ± 0.09a
Car/ Chl	6.31 ± 0.04b	7.02 ± 0.16a	6.51 ± 0.10b	6.45 ± 0.08b
Stomatal density in upper epidermis [mm^{-2}]	190 ± 3a	190 ± 3a	214 ± 4b	194 ± 3a
Stomatal density in lower epidermis [mm^{-2}]	167 ± 2d	199 ± 3c	239 ± 4a	215 ± 3b
Stomatal length in upper epidermis [μm]	26.3 ± 0.3a	24.5 ± 0.1b	24.1 ± 0.1bc	23.8 ± 0.1c
Stomatal length in lower epidermis [μm]	27.5 ± 0.2a	27.2 ± 0.2a	26.6 ± 0.2b	24.9 ± 0.2c
The upper epidermis thickness [μm]	17.2 ± 0.8b	21.7 ± 0.7a	21.8 ± 0.6a	20.7 ± 0.5a
The lower epidermis thickness [μm]	17.4 ± 0.5a	16.5 ± 0.5a	16.3 ± 0.5a	16.2 ± 0.5a
The palisade tissue thickness [μm]	7 4.6 ± 2.1c	98.9 ± 2.8b	95.4 ± 1.8bc	102.9 ± 2.4a
The spongy tissue thickness [μm]	43.7 ± 1.9a	42.2 ± 1.5a	44.8 ± 1.4a	46.7 ± 1.4a
The palisade to spongy thickness ratio	1.85 ± 0.11b	2.5 ± 0.12a	2.23 ± 0.08a	2.34 ± 0.11a
The lamina thickness [μm]	152.8 ± 2.6b	179.3 ± 3.6a	178.3 ± 2.5a	186.4 ± 2.8a

photosystems become impaired as the stress increases (Gomes *et al*. 2008, Vaillant *et al*. 2005) while dark respiration or photorespiration might be increased (Chaney 1993, Hura *et al*. 2007, Monnet *et al.* 2001, Van Assche and Clijsters 1986b). This deserves further study.

In conclusion, Zn exposure inhibits photosynthesis and transpiration, reduces root biomass and pigment content, and results in xerophyte structure in leaves. The changes in the leaf structure, chlorophyll and carotenoid contents, and stomatal conductance are responsible for the decline of photosynthetic rate caused by Zn.

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