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Zn-localization and anatomical changes in leaf tissues of green beans (*Phaseolus vulgaris* L.) following foliar application of Zn-lignosulfonate and ZnEDTA

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A B S T R A C T

Understanding the effective penetration of Zn fertilizers, following foliar application, requires powerful techniques to trace over a period of time the pathway of Zn within the leaf. In this paper, atomic absorption spectroscopy, Cryo-scanning electron microscopy (Cryo-SEM) and energy-dispersive X-ray microanalysis (CEDX) was used to study Zn uptake and localization at leaf cellular level, at different times after the foliar-application of a Zn-lignosulfonate (Zn-LS) complex in comparison with a ZnEDTA chelate.

Green bean plants (*Phaseolus vulgaris* L., cv. Linera) were grown in pots and Zn-LS and ZnEDTA fertilizers were compared in foliar application on the oldest leaves. Zinc concentration in *Treated Non-treated leaves* and "washing away" water was determined by atomic absorption spectrophotometry at 6 h, 4 and 30 days. Significant differences in Zn penetration inside the leaves were observed, the most efficient being the Zn LS complex which showed fastest absorption after 6 h. At this time, also significant differences in Zn localization inside the leaf tissues were detected by CEDX, the mesophyll showing the highest absorption of Zn LS. Zn were detected in the mesophyll at the highest concentration in leaves of plants treated with Zn-LS also at the 4th and 30th days, whereas in those treated with ZnEDTA it was in the lower epidermis. The analyses of structural changes on frozen-hydrated leaf samples by Cryo-SEM showed that Zn-LS complex treatment causes an increase of the total thickness of the leaf and in particular of the spongy mesophyll, the innermost and physiologically active tissue layer.

1. Introduction

Zinc (Zn) is a micronutrient deficiency of which limits crop productivity worldwide (Fageria, 2009). Moreover, Zn deficiency is a widespread nutritional disorder affecting human health, especially in those regions of the world where staple food crops are the main source of daily calorie intake (Graham et al., 1999). For these reasons, many efforts have been made in improving Zn fertilization of crops (Fageria, 2009; Sadeghzadeh and Rengel, 2011).

Beside soil Zn fertilization, foliar application of Zn fertilizer has been shown to be effective for delivering Zn to plant while enriching food crop with the desirable amounts of Zn for human nutrition (Ram et al., 2016). Foliar application is a short-term methodology, which provides more efficient use of nutrients remediating plant mineral nutrient deficiencies in short time than soil application (Fernández et al., 2013). Indeed, Zn is classified as an intermediate or conditionally mobile element (Marschner, 2012). The mechanisms by which foliar applied products penetrate into leaf surface are influenced by polar nature and size of the applied molecules (Fernández and Brown, 2013). Moreover, plant response to foliar fertilization in the field is an extremely complicated process, which depends by several variables including the environmental factors (Fernández et al., 2013).

A large number of Zn fertilizers are available for foliar application and increasing evidences prove that in field conditions, foliar applications maximize uptake and accumulation of Zn (Cakmak, 2008; Pandey et al., 2013). However, not all zinc-containing sprays seem to be equally effective (Benedicto et al., 2011). Some studies have proved that foliar-applied Zinc disodium ethylenediaminetetraacetate (ZnEDTA) is suitable for providing adequate Zn nutrition to plants (Karak et al., 2005) while others reported that ZnEDTA did not improve significantly the Zn concentration in the leaves after its foliar application (Wei et al., 2012). Lignosulfonates are by-products obtained from the pulp and paper

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Table 1
Plant growth of green bean plants after 30 days from the treatments (control, Zn-LS complex and ZnEDTA). Data (n = 5), were analysed with ANOVA analysis at 0.05 probability level. Values are means ± standard deviation. *ns*, not significant. Height (cm), Leaf number (n), Fresh weight (g), LA, leaf area (m^2); SLA = specific leaves area ($m^2\ kg^{-1}$), LAR = leaf area ratio ($m^2\ kg^{-1}$), LMR = leaves mass ratio ($kg_{leaf}^{-1}\ kg_{plants}^{-1}$).

		Control	Zn-LS	ZnEDTA	ANOVA
Plant	*Height*	26 ± 5	26 ± 4	26 ± 6	ns
	Leaf number	21 ± 4	23 ± 6	21 ± 3	ns
	Fresh weight	20.5 ± 7.86	21.9 ± 6.03	24.1 ± 2.31	ns
Beans	*Numbers*	4 ± 1.5	4 ± 2.1	5 ± 1.6	ns
	Fresh weight	1.3 ± 0.65	1.4 ± 0.56	1.4 ± 0.41	ns
	Lengths	6.5 ± 1.93	7.9 ± 1.85	6.9 ± 2.37	ns
Treated leaves	*LA*	131 ± 48.6	206 ± 95.7	209 ± 26.2	ns
	SLA	679 ± 315	1064 ± 515	712 ± 58	ns
	LAR	68 ± 22	101 ± 33	76 ± 11	ns
	LMR	0.100 ± 0.03	0.100 ± 0.03	0.093 ± 0.02	ns
Non-treated leaves	*LA*	581 ± 276.8	856 ± 520.9	843 ± 201.9	ns
	SLA	915 ± 212	905 ± 202	833 ± 160	ns
	LAR	335 ± 39	403 ± 260	324 ± 67	ns
	LMR	0.337 ± 0.11	0.37 ± 0.012	0.390 ± 0.04	ns

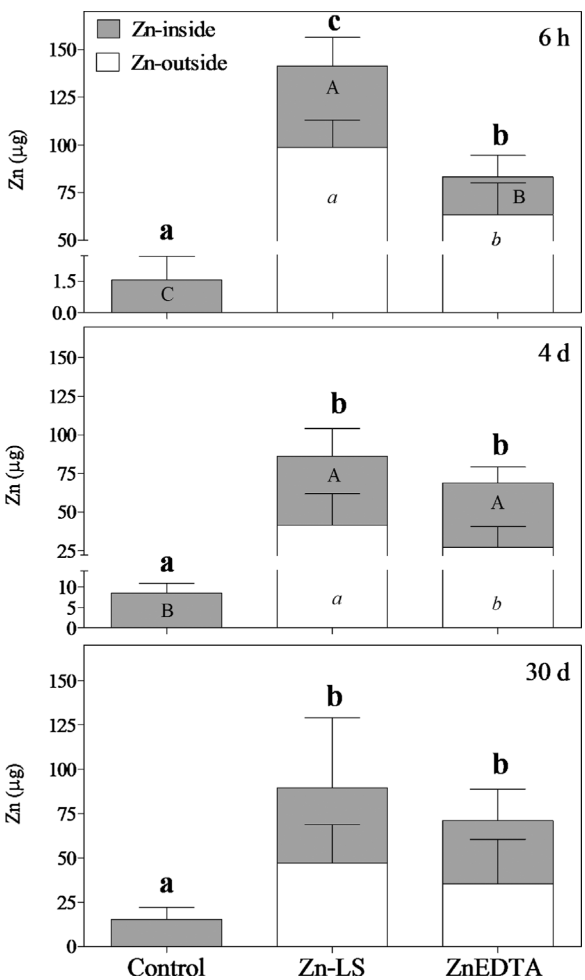

Fig. 1. Content of *Zn-inside and -outside* ("washing away" water) leaves at 6 h, 4 days and 30 days after Zn-LS complex and ZnEDTA treatments. Data of *Zn-inside*, and Zn-total = *Zn-outside* + *Zn-inside* (n = 5) were subjected to one-way ANOVA and the different letters (*Zn-inside – UPPERCASE,* Zn-total – **bold**) represent differences among means following Bonferroni Test (P ≤ 0.05). Data of *Zn-outside* were subjected to Student's t-*test* and the different letters (*Italics*) represent differences at 0.05 probability level.

industry and are used as complexing agents in several commercial Zn formulations (Martın-Ortiz et al., 2009). These Zn organic complexes are eco-compatible and less expensive alternatives to synthetic chelates (Benedicto et al., 2011; Fernández et al., 2013), although in the past some Authors reported that in soil Zn-lignosulfonates (Zn-LS) products are less effective than chelates because of the weak bonds between the metal and the complexing agent (Gangloff et al., 2002; Álvarez and Gonzalez, 2006).

Measurements of mineral element concentrations in tissues or single cells can be done on frozen-hydrated plant organs, by Cryo-scanning electron microscopy (Cryo-SEM) (McCully et al., 2009) coupled with energy dispersive X-ray microanalysis (CEDX) (McCully et al., 2010). This is highly relevant when comparing spray fertilizers effectiveness in delivery micronutrients inside plant organs.

Aim of this study was to verify the Zn-LS effectiveness in comparison with a synthetic chelate (ZnEDTA) fertilizer when applied to leaves of green bean, in terms of Zn uptake, localisation and translocation into leaf tissues.

2. Material and methods

2.1. Plant materials and Zn treatments

Green bean (*Phaseolus vulgar* L., cultivar "Linera") seeds were germinated in the dark at 30 °C on filter paper moistened with distilled water. After germination (7 ± 2 days), seedlings were transferred into 1.4 dm^3 pots with a growth medium (VIV MC, VIGORPLANT Italia srl, Italy) composed by Irish and Baltic peats (pH 6.0; EC 0.30 dS/m; dry bulk density 170 kg/mc; total porosity: 90%), and then placed in a growth chamber at 21 ± 2 °C, 70 ± 5% of relative humidity and an irradiance of 1000 $\mu mol\ m^{-2} s^{-1}$ provided during 16-h photoperiod. After 23 days of acclimation, forty-five homogeneous plants were sorted out for the trials, and randomly divided into 3 groups. The following spray treatments were applied to plants of each group in a single occasion at the XV phenological growth stage (according to BBCH classification):

- Control (milliQ water containing 0 mg L^{-1} Zn).
- Zn-LS complex (milliQ water containing 150 mg L^{-1} Zn, Brexil Zn®-lignosulfonate-based Zn formulation, Valagro proprietary formulation). Chemical characteristics: pH 3.5 (1% solution); organic-S (4% dry weight); phenolic-OH (0.8% dry weight).
- ZnEDTA chelate (milliQ water containing 150 mg L^{-1} Zn, Zinco EDTA 75).

All treatments were delivered in a volume of 150 ml that was manually sprayed on the first two developed three-lobe leaves (*Treated-leaves*). Spray application was carried out on both leaf sides without wetting the rest of the plant organs. The average amount of liquid retained by the two treated leaves was 2 ± 1.3 ml.