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RESEARCH ARTICLE

Xylem sap mineral analyses as a rapid method for estimation plant-availability of Fe, Zn and Mn in carbonate soils: a case study in cucumber

Nikolai Bityutskii*, Kirill Yakkonen, Anastasiya Petrova, Marina Nadporozhskaya

*Department of Agricultural Chemistry, Saint Petersburg State University, Universitetskaya nab., 7/9, Saint Ptersburg, 199034, Russia. *Corresponding author: n.bityutskii@spbu.ru; bityutskii@mail.ru*

Abstract

Low plant-availability of iron (Fe), zinc (Zn) and manganese (Mn) leads to micronutrient deficiency, causing significant yield reductions of crops throughout the world, especially in calcareous soils. This study was performed in order to evaluate the efficiency of xylem sap analysis in the determination of Fe, Zn and Mn availability in plants (*Cucumis sativus* L.) affected by calcium carbonate ($CaCO_3$) levels. A soil with six levels of $CaCO_3$ (0−10% DW) was used. We performed a combination approach, including analysis of the soil mobility of micronutrients using different extractants (water, DTPA-TEA and ammonium acetate), as well as xylem and shoot elemental analysis. Generally, application of $CaCO_3$ resulted in a pH increase of the bulk soil of 1.4−2.2 pH units; extractability of all micronutrients was significantly decreased 1.4−4.2 times, irrespective of the extracting solution. Xylem sap Fe, Zn and Mn concentrations were significantly correlated with the respective concentrations in the soil extracting solutions. By contrast, only shoot concentrations of Zn and Mn, but not of Fe, were linearly correlated with their extractable forms. With electrothermal atomic absorption spectrometry, changes in xylem sap concentrations of micronutrients were detected without preliminary mineralization of plant material, in contrast to shoot analysis. Our results demonstrate that xylem sap analysis offers the advantages of a simple characterization of multi-microelement availability in plants under $CaCO_3$ stress.

Keywords: Availability, calcium carbonate, cucumber, iron, manganese, xylem sap, zinc

1. Introduction

Iron (Fe), zinc (Zn) and manganese (Mn) are nutrients required by all living organisms, including higher plants. Iron is a component of a number of enzymes functioning as an electron carrier in metabolic processes such as respiration and photosynthesis, Fe is involved in the synthesis of chlorophyll and essential for the maintenance of chloroplast structure and function (Marschner, 1995), and Zn is an essential component of many enzymes and a structural stabilizer of proteins and plant membranes (Broadley *et al*., 2007). Manganese plays a role in the charge accumulating process in the active site in the water-splitting system of photosystem II (PSII) (Goussias *et al*., 2002) and acts as a cofactor activating many different enzymes (Marschner, 1995).

Although abundant in most agricultural soils, bioavailability of Fe, Zn and Mn is often limited by calcium carbonate ($CaCO_3$), especially in plants grown in calcareous soils. In such soils, $CaCO_3$ is a major component which buffers soil solution pH at 7.5−8.5, due to a high bicarbonate concentration (HCO_3^-) (Lindsay, 1995). It is generally thought that soil pH governs plant-availability of many soil microelements, including Fe, Zn and Mn, and distinct effects of the prevailing rhizosphere pH on the availability of these three micronutrients were found (Sarkar and Wyn Jones, 1982; Rengel, 2015). Ferric (Fe^{3+}) and ferrous (Fe^{2+}) activity in the soil solution decrease 1,000-fold and 100-fold, respectively, for each unit increase in soil pH (Lindsay, 1995). The low bioavailability of Fe, Zn and Mn at moderate and high pH leads to deficiency of these micronutrients, causing significant yield reductions of crops throughout the world (Alloway, 2008). Calcareous soils with excess $CaCO_3$ represent one third of the world's agricultural soils (Vose, 1982), and corrections of low pH by liming are common amendments for acid soils (Monfort-Salvador *et al*., 2015). Further alkalization of agricultural soils causes by common high-input farming practices such as the extensive use of biomass-ash (Quirantes *et al*., 2016). To examine nutrient availability before seed sowing, soil extraction methods are widely used (Lindsay and Norvell, 1978; Wang *et al*., 2004; Fonseca *et al*., 2010), enabling the detection of nutrients which can be potentially absorbed by plants. However, to monitor the actual nutritional status of plants, chemical nutrient analysis has been commonly used (Abadia *et al*., 2004; Chatterjee and Dube, 2004). After acquisition by roots, nutrients are transported to the shoots through xylem vessels. The vascular system is an essential segment for long distance transport of nutrients in plants. Therefore, xylem sap analysis has been used to evaluate both nutrient availability in soil and the nutritional status of plants (Stark *et al*., 1985; Noguchi *et al*., 2005ab).

Recently, methods for xylem sap sampling and determination of micronutrient species involved in long distance translocation have been successfully reviewed (Alexou and Peuke, 2013; Álvarez-Fernández *et al*., 2014). However, information on the relevance of xylem sap analysis to evaluate Fe, Zn and Mn plant-availability affected by $CaCO_3$ levels is still lacking, partly due to the fact that methods of evaluating nutrient availability in soils using xylem sap have so far mainly been applied under conditions in which $CaCO_3$ was not present. In such experiments, nutrient availability was changed by changing the total nutrient amount in a nutrient solution (Alam *et al*., 2001), a sand culture medium (Noguchi *et al*., 2005a) or by controlling the cation exchange capacity (CEC) of a soil (Noguchi *et al*., 2005b). To change the CEC value, a humic acid reagent and a synthetic zeolite were used (Noguchi *et al*., 2005b). Moreover, limited information is available on how xylem sap reflects

Fe availability for higher plants subjected to excess $CaCO_3$, taking into account that plants may develop specific physiological and morphological responses at the root level to overcome Fe deficiency (Römheld and Marschner, 1986; García-Mina *et al.*, 2013). The effects of Fe deficiency on xylem sap composition have been characterized when plants (sugar beet) were grown in a nutrient solution (not in a soil) buffered by adding $CaCO_3$ (López-Millán *et al.*, 2000). Very recently, changes in xylem sap concentrations of Ca^{2+} have been investigated in order to test the hypothesis that elevated xylem sap Ca^{2+} concentration is responsible for observed reduction in gas exchange of legumes grown in limed soil (Rothwell and Dodd, 2014). In addition, in cucumber (which was also used in this study) xylem sap concentrations of microelements, including Fe, Zn and Mn, were influenced by NO_3: NH_4 ratio, but not by $CaCO_3$ levels (Zornoza and Carpena, 1992).

The aim of this study was to investigate how concentrations of Fe, Zn and Mn in the xylem sap reflect the availability of these microelements affected by different $CaCO_3$ levels. In this study, we hypothesized that xylem sap can potentially be used to evaluate availability of Fe and other microelements (i. e. Zn and Mn) in soils with different $CaCO_3$ contents.

2. Materials and Methods

2.1. Soil material

The soil (Anthri-Umbri-Endogleyic Luvisol) was collected from the humus layer of permanent grassland near the Biological Research Institute of Saint Petersburg State University, Petergof, Russia. The main chemical soil characteristics were a pH in water of 5.7, 3.36% total C, 0.18% total N, and 40 and 105 mg kg^{-1} of dry soil available P and K respectively. For the $CaCO_3$ treatments, 0, 0.1, 0.5, 1.0, 5.0 and 10.0% dry soil of calcium carbonate in the form of fine powder was added to the soil. The soils (+/−$CaCO_3$) were pre-incubated at 60% water holding capacity (WHC) for one week before seed sowing. This experimental setting seems suitable as a model to mimic the natural conditions of both calcareous and limed soils.

2.2. Plant material and growth conditions

Three cucumber plants (*Cucumis sativus* L., cv. Semcross) were grown in each plastic pot filled with 1 kg soil (dry weight) for 34 days. Cucumber is one of the most widespread horticultural crops and about 90% of cucumber plants are cultivated using alkaline soils with a relatively high $CaCO_3$ content (Bacaicoa and García-Mina, 2009). Plants were grown in a room at 24 ± 2ºC: 20 ± 2ºC (light: dark), with a day/night regime of 16/8 h and photon flux density of 200 μmol m^{-2} s^{-1} at plant height. The plants were irrigated daily with distilled water to keep soil moisture at about 60 % WHC. Plants were harvested at 20 and 34 days after seed sowing.

2.3. Plant analysis

Xylem sap was collected by a micropipette 1 h after stems were cut 2 cm above the root base, after discarding exudates obtained during the first few minutes. Concentration of Fe, Zn and Mn in freshly collected xylem sap was quantified immediately after the samples were diluted by electrothermal atomic absorption spectrometry (GFAAS; model MGA 915, Lumex, Russia) with Zeeman-effect background correction, equipped with graphite tubes (Schunk, Germany). The dilution rates were variable and depended on the original concentration. Xylem sap translocation rate of micronutrients was calculated as sap micronutrient concentration × exudate rate.

At harvest, shoots of plants were thoroughly washed with distilled water, dried at 70 ºC for 48 h and weighed. Dry shoot material (0.1 g) was digested in concentrated HNO_3 in a microwave (microwave digestion system; model Minotavr-2, Lumex, Russia). The concentrations of micronutrients in shoots were also quantified by GFAAS.

2.4. Soil analyses

The pH in water (pHw) was measured in a 1: 2.5 soil-water solution with a glass electrode. Total carbon (C) and nitrogen (N) contents were determined using a CHN-analyzer (model CHN-628, Leco Corporation, USA). Available P and K were measured in a 1: 5 soil–0.2 N HCl solution. Soil available contents of Fe, Zn and Mn were extracted using the following procedures:

1) Method-1 [H_2O (10: 1 extractant/fresh soil ratio)]. Soil samples were shaken for 1 h in water.
2) Method-2 [DTPA-TEA solution (5: 1)]. Soil samples were shaken for 2 h with DTPA-TEA (0.005 M diethylenetriaminepentaacetic acid + 0.1 M triethanolamine + 0.1 M $CaCl_2$) at pH 7.3 (Lindsay and Norvell, 1978). This method is used as a multielement extraction method in various regions of the world.
3) Method-3 [AA solution (10: 1)]. Soil samples were shaken for 1 h with ammonium acetate (1 M, pH 4.8) (Mineev *et al.*, 2001).

This procedure is widely used in various regions of the world for a large range of soils, including calcareous soils.

To prevent a possible decrease in micronutrient mobility induced by soil drying, fresh soil samples were used for the extractions. Micronutrient concentrations in soil extracts were determined by GFAAS and expressed on a dry soil weight basis.

2.5. Statistical analysis

All statistical analyses were performed using IBM SPSS Statistics version 21.0. Data were subjected to analysis of variance procedures (ANOVA, type III) and means were compared by Student-Newman-Keuls's post-hoc test at 5% significance level ($P < 0.05$). Four replicate pots were used for each treatment. The residuals of each model were analysed to test for normality of variance. The data were inspected for homogeneity of variance (Levene test). To test whether parameters characterizing soil mobility and availability of micronutrients were correlated, Pearson coefficients (r) were determined.

3. Results

3.1. Effect of $CaCO_3$ on soil pH and micronutrient mobility

Generally, application of $CaCO_3$ resulted in a pH increase of the bulk soil of 1.4−2.2 pH units (Figure 1); extractability of all micronutrients was significantly decreased 1.4−4.2 times, irrespective of the extracting solution (Figure 2).

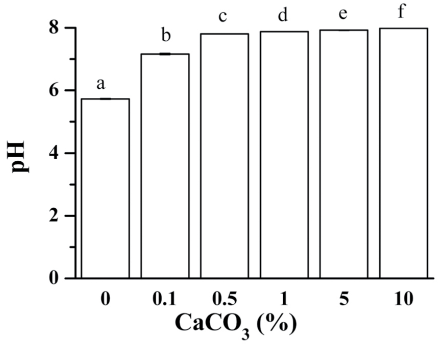

Figure 1. Water pH of soil with $CaCO_3$. Data represent means ± SD. Significant differences between treatments ($P < 0.05$, $n = 4$) are indicated by different letters.

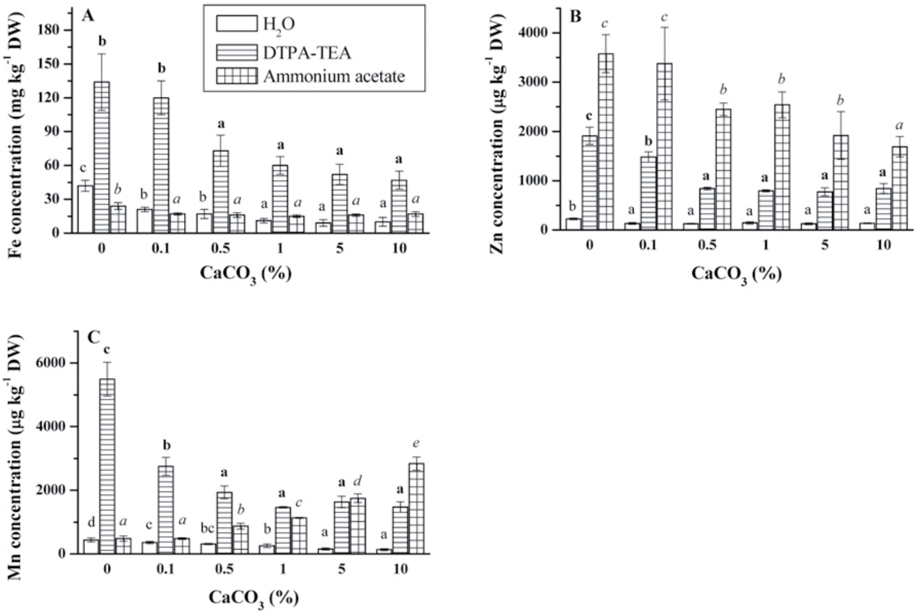

Figure 2. Concentrations of Fe (A), Zn (B) and Mn (C) extracted with water, DTPA-TEA or ammonium acetate in soil with $CaCO_3$. Data represent means ± SD. Significant differences between treatments ($P < 0.05$, $n = 4$) are indicated by different letters.

Only one unusual effect associated with liming was observed: an increase of the soil Mn concentrations when extracted with ammonium acetate (pH 4.8) at $CaCO_3 \geq 0.5\%$ (Figure 2C). This may be a result of solubilisation of some impurities of Mn within $CaCO_3$. Indeed, the amount of Mn extracted by ammonium acetate from $CaCO_3$ fine powder (166 mg kg^{-1}) was 33 times higher than that extracted by DTPA-TEA solution (5 mg kg^{-1}). In contrast, Mn extracted from $CaCO_3$ by water was not detectable.

3.2. Plant performance

Dry biomass of cucumber tended to decrease with increased $CaCO_3$ rates (Figure 3). A significant decrease ($P < 0.05$) of plant biomass by 13−17% was recorded at $CaCO_3$ contents ≥ 1% in comparison to the $-CaCO_3$ soil.

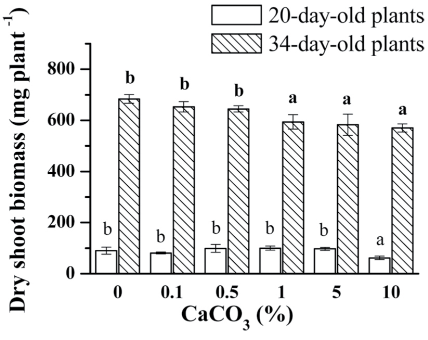

Figure 3. Dry biomass of *Cucumis sativus* grown in soil with $CaCO_3$. Data represent means ± SD. Significant differences between treatments ($P < 0.05$, $n = 4$) are indicated by different letters.

Addition of $CaCO_3$ significantly lowered Fe, Zn and Mn concentrations in xylem sap (by 1.6−3.6 times), especially in 20-day-old plants in comparison with plants that had not received $CaCO_3$ (Figure 4).

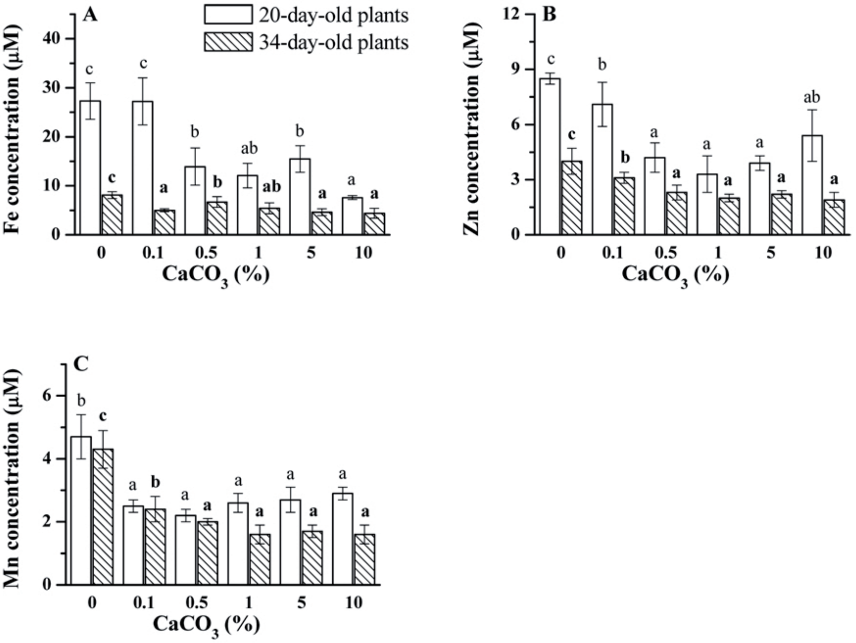

Figure 4. Xylem sap concentrations of Fe (A), Zn (B) and Mn (C) in *Cucumis sativus* grown in soil with $CaCO_3$. Data represent means ± SD. Significant differences between treatments ($P < 0.05$, $n = 4$) are indicated by different letters.

Translocation rates of Zn and Mn via xylem (nmol plant^{-1} h^{-1}) significantly decreased (by 2−2.6 times) after $CaCO_3$ application while the rate of Fe was not altered by $CaCO_3$ (data not shown). Shoot concentrations of Zn and Mn decreased with liming on average 1.9 and 1.4 times, respectively (Figure 5B, C), but shoot concentrations of Fe were not significantly affected by $CaCO_3$ treatment (Figure 5A).

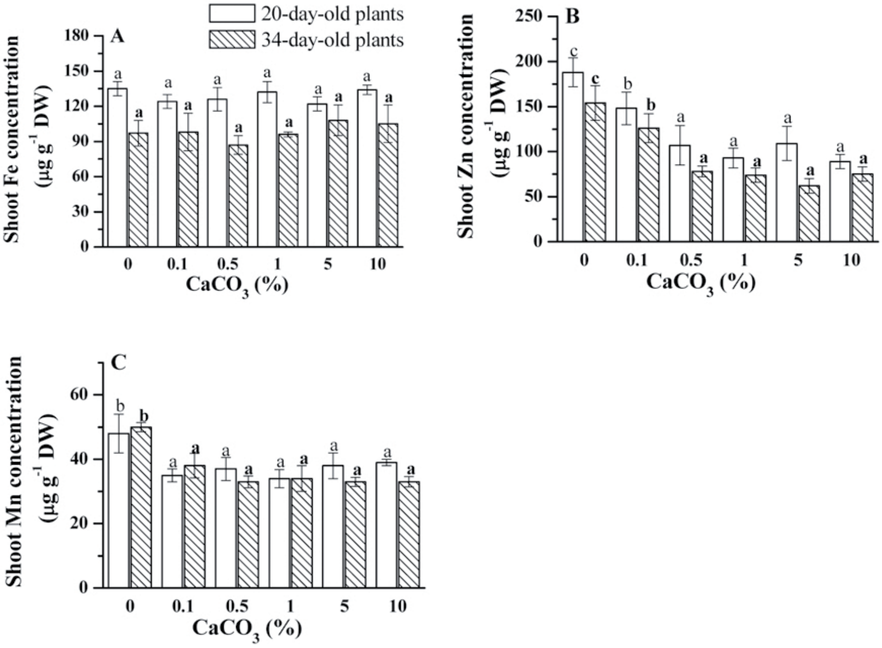

Figure 5. Shoot concentrations of micronutrients Fe (A), Zn (B) and Mn (C) in *Cucumis sativus* grown in soil with $CaCO_3$. Data represent means ± SD. Significant differences between treatments ($P < 0.05$, $n = 4$) are indicated by different letters.

3.3. Correlations

Xylem sap Fe concentrations were strongly positively correlated with concentrations of soil extractable Fe, especially for Fe extracted by water and DTPA-TEA solution (Table 1).

However, Pearson´s correlation coefficient between shoot Fe and soil extractable Fe was not significant at $P < 0.05$, irrespective of extracting solutions. Soil extractable Zn (especially DTPA-extractable Zn) was correlated with both xylem sap Zn and shoot Zn ($P < 0.01$). Generally, soil Mn concentrations extracted by water and especially by DTPA-TEA solution were positively correlated with both xylem sap Mn and shoot Mn ($P < 0.01$). Pearson´s correlation coefficients between xylem sap/shoot Mn and soil Mn extracted with ammonium acetate were not significant or negative (Table 1).

Table 1. Pearson coefficients (r) calculated for parameters characterizing soil mobility of Fe, Zn and Mn and availability of these micronutrients to *Cucumis sativus*

	Fe						
	FeH$_2$O	FeDTPA	FeAA	XSFe 1	XSFe 2	SFe1	SFe 2
FeH$_2$O	1						
FeDTPA	**0.804****	1					
FeAA	**0.830****	**0.627****	1				
XSFe 1	**0.717****	**0.753****	**0.500***	1			
XSFe 2	**0.698****	**0.502****	**0.549****	0.377	1		
SFe 1	0.166	–0.076	0.263	0.008	0.236	1	
SFe 2	–0.136	–0.147	–0.023	0.005	–0.241	–0.048	1
	Zn						
	ZnH$_2$O	ZnDTPA	ZnAA	XSZn 1	XSZn 2	SZn 1	SZn 2
ZnH$_2$O	1						
ZnDTPA	**0.707****	1					
ZnAA	**0.444***	**0.767****	1				
XSZn 1	**0.607****	**0.767****	**0.652****	1			
XSZn 2	**0.725****	**0.907****	**0.688****	**0.676****	1		
SZn 1	**0.668****	**0.883****	**0.698****	**0.640****	**0.838****	1	
SZn 2	**0.718****	**0.904****	**0.792****	**0.794****	**0.797****	**0.836****	1
	Mn						
	MnH$_2$O	MnDTPA	MnAA	XSMn 1	XSMn 2	SMn 1	SMn 2
MnH$_2$O	1						
MnDTPA	**0.747****	1					
MnAA	**–0.849****	**–0.579****	1				
XSMn 1	**0.460***	**0.782****	–0.216	1			
XSMn 2	**0.714****	**0.964****	**–0.581****	0.337	1		
SMn 1	0.304	**0.699****	–0.122	**0.781****	**0.695****	1	
SMn 2	**0.678****	**0.907****	**–0.514***	**0.814***	**0.904****	**0.683****	1

H_2O – soil concentration of micronutrient (i. e. Fe, Zn or Mn) extracted by water. DTPA – soil concentration of micronutrient extracted by DTPA-TEA solution at pH 7.3. AA - soil concentration of micronutrient extracted by ammonium acetate at pH 4.8. XS 1 and 2 – xylem sap concentration of micronutrient in 20-day-old (1) and 34-day-old (2) plants. S 1 and 2 – shoot concentration of micronutrient in 20-day-old (1) and 34-day-old (2) plants. Significant effects are given in bold. * $P < 0.05$. **, $P < 0.01$

4. Discussion

We tested the hypothesis whether the xylem sap method can effectively be used to predict Fe, Zn and Mn availability as affected by $CaCO_3$ levels. Overall, $CaCO_3$ addition increased soil pH (Figure 1) and led to a significant decrease (by 1.4−4.2 times) in soil solubility of all micronutrients extracted by different methods (i.e. water, DTPA-TEA or ammonium acetate) (Figure 2). $CaCO_3$ buffers soil solution pH at 7.5−8.5 due to a high bicarbonate concentration (Lindsay, 1995). The presence of bicarbonate and high pH in the soil cause a diminished bioavailability of some nutrients, especially Fe, Zn and Mn (Rengel, 2015). The metals Fe and Mn are transition metals with insoluble hydroxides (Lindsay, 1995), whereas Zn sorbs to the surface of calcites, influencing their aqueous-phase concentrations in calcareous environments (Kitano *et al*., 1976). In our study, only Mn extracted by ammonium acetate (pH 4.8) increased with elevated rates of $CaCO_3$: from 0.5 to 10% (Figure 2C). Although ammonium acetate is used as an indicator of effectiveness of exchangeable forms, in this study, the solution did not necessarily give results corresponding to those of Mn in soils with relatively high $CaCO_3$ levels. It seems that ammonium acetate (pH 4.8) dissolved significantly larger amounts of Mn impurities within $CaCO_3$ as compared with both water and DTPA-TEA extractants.

The xylem sap method had a distinct advantage in the prediction of plant availability of the investigated micronutrients limited by $CaCO_3$. The concentrations of Fe, Zn and Mn in xylem sap decreased linearly (by 1.6–3.6 times) with decreased extractability induced by $CaCO_3$ applications (Figure 4). There was a significant correlation between xylem sap concentrations of the micronutrients and their extracted amounts for both 20-day-old and 34-day-old cucumber plants (Table 1). Thus, xylem sap analysis was effective to predict availability of Fe, Zn and Mn at an early stage of plant growth (20-day-old plants after seed sowing), even if symptoms of micronutrient disorders (a decrease in plant biomass) were not yet visible (e.g. at 0.1 and 0.5% $CaCO_3$) (Figure 3).

The same trends could be observed for shoot concentrations of Zn and Mn (Figure 5B, C), whereas distinct effects of $CaCO_3$ on shoot Fe concentrations were not found (Figure 5A), despite the fact that applications of $CaCO_3$ lowered both soil extractable Fe and xylem sap Fe (Figures 2 and 4A). In plants with limited leaf growth, total Fe concentration in chlorotic leaves can be similar or even higher when compared to green ones, indicating the so-called "chlorotic paradox". This effect seems a consequence of the diminished dilution of the normal Fe concentration in leaves (Römheld, 2000). An alternative explanation for the "chlorosis paradox" is the existence of Fe pools which have precipitated in the leaf (Jiménez *et al*., 2009). On the other hand, whilst the xylem sap Fe concentrations decreased with elevated $CaCO_3$ contents, the translocation rate of Fe via xylem was not affected by those treatments (data not shown). To overcome Fe deficiency, plants develop specific mechanisms: Strategy I and II (Marschner, 1995). Strategy I is a complex Fe uptake mechanism developed by dicots and non-grass monocots. Strategy I plants respond to Fe deficiency by releasing H^+ and organic compounds (e. g. phenols, flavins and organic acids) in rhizosphere and enhancing of a Fe^{3+}-chelate reductase (FC-R) at the plasma membrane of the rhizodermal cells (Römheld and Marschner, 1986; García-Mina *et al.*, 2013). Proton extrusion and the release of organic compounds could help in the solubilization of Fe compounds. Once reduced, the Fe^{2+} ions are transported in the roots by a carrier (IRTs) belonging to the ZIP family of transporters (Guerinot, 2000). Strategy II plants (other gramineous plants) secrete phytosiderophores – Fe-chelating substances that solubilize Fe in soils (Römheld and Marschner, 1986; García-Mina *et al*., 2013). However, it has been demonstrated that HCO_3^- can block the expression of ferric reductase (*FRO*), H^+-ATPase (*HA1*) and iron transporter (*IRT1*) genes, as well as the activity of the corresponding enzymes (FC-R, H^+-ATPase) in roots of Strategy I plants (Lucena *et al.*, 2007).

Cucumber plants are able to activate the main Fe-stress root responses of Strategy I plants: rhizosphere acidification, Fe reduction and specific morphological changes (Bacaicoa and García-Mina, 2009). The roots and leaves of cucumber responded to the lack of Fe by increasing citrate tissue concentration (Bityutskii *et al*., 2014). Citrate has been considered the most likely major candidate for xylem Fe transport (Rellán-Álvarez *et al.*, 2010). By contrast, little is known about specific mechanisms responsible for Zn or Mn deficiencies in plants. In this study, Zn and Mn, but not Fe, translocation rates via the xylem significantly decreased with liming (data not shown). In a very recent study, it could be shown that Zn and Mn deficiency does not increase the root and leaf concentrations of citrate and some other organic acids (Bityutskii *et al*., 2014).

Micronutrient concentrations were determined by the widely used electrothermal atomic absorption spectrometry. This method allows to use a smaller amount of xylem sap without preliminary mineralization of the sap using concentrated acid (HNO_3) and microwaving, in contrast to the traditional analysis of plant materials (shoots, leaves and others). In perspective, xylem sap analysis is an important method especially for the monitoring of the actual nutritional status of plants grown on calcareous and limed soils which generally contain large quantities of total micronutrients, but only very small quantities of many micronutrients in soluble forms.

Conclusions

Among the different available plant-tests (i.e. xylem sap analysis and shoot analysis), only xylem sap analysis offers the advantages of a simple characterization of multi-microelement availability in $CaCO_3$-stressed plants. In our study, concentrations of the micronutrients Fe, Zn and Mn in xylem sap were directly proportional to those in the soil extracting solutions, irrespective of the vegetative phase of the growth period. With electrothermal atomic absorption spectrometry, changes in xylem sap concentrations of micronutrients due to a variation in their availability may be detect without preliminary mineralization of plant material, as compared with shoot analysis. Xylem sap analysis could effectively be used to predict Fe, Zn and Mn plant availability even if symptoms of micronutrient disorders are not yet visible.

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