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BRIEF COMMUNICATION

Effects of cadmium on gas exchange and phytohormone contents in citrus

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Abstract

The effect of increased Cd^{2+} concentrations in the watering solution on citrus physiology was studied by using two citrus genotypes, Cleopatra mandarin and Carrizo citrange. Cadmium content in roots and leaves was tested together with measurements of leaf damage, gas exchange parameters, and hormonal contents. Citrus roots efficiently retained Cd^{2+} avoiding its translocation to the shoots and Cleopatra mandarin translocated less Cd^{2+} than Carrizo. With increasing Cd^{2+} concentration all gas exchange parameters were decreased more in Carrizo than in Cleopatra mandarin. Cd-induced increases in abscisic acid and salicylic acid contents were observed in leaves but not in roots of both genotypes.

Additional key words: abscisic acid, net photosynthetic rate, jasmonic acid, salicylic acid, stomatal conductance, transpiration rate.

Cadmium ions are easily absorbed by plant roots. In some species Cd^{2+} remains in roots (Rascio *et al.* 2008, Jiang *et al.* 2009) whereas in other species, such as tobacco, Cd^{2+} is translocated to the leaves. Nevertheless, the Cd^{2+} content is usually much higher in roots than in leaves (Wagner 1993). Grafting experiments have been performed in *Nicotiana* species indicating that the responsible mechanisms for limited translocation are located in the roots (Lugon-Moulin *et al.* 2004). In the case of citrus, scarce information can be found on the ability of plants to translocate Cd^{2+} from roots to aerial parts.

The symptoms of Cd^{2+} toxicity are well documented in several herbaceous species (Di Toppi and Gabbrielli 1999, Jiang *et al.* 2009). Visible effects of exposure to the high metal doses are growth inhibition and leaf chlorosis. The photosynthetic apparatus, the water balance and the stomatal opening are also seriously disturbed (Rascio *et al.* 2008). Oxidative stress has often been discussed as a primary effect of Cd^{2+} exposure (Markovska *et al.* 2009, Rodriguez-Serrano *et al.* 2009). Recently, it has been shown that microtubular cytoskeleton is one of the targets of Cd toxicity in root tip cells (Xu *et al.* 2009). Among responses to Cd^{2+}, synthesis of phytochelatins has been extensively studied (Ben Ammar *et al.* 2008) and constitutes a specific plant response to metal toxicity. Another group of well-known responses is the up-regulation of stress proteins (Schutzendubel *et al.* 2001) or the accumulation of proline (Schat *et al.* 1997). Only a few studies have indirectly implicated the stress hormones jasmonate and ethylene in the transcriptional control of pathogen-related and heat shock proteins under Cd^{2+} excess, probably to protect cells against damage induced by Cd^{2+} treatment (Rodriguez-Serrano *et al.* 2009).

Despite the lack of studies on the effect of Cd^{2+} on citrus physiology, the response of this crop to other environmental stresses has been extensively studied. It is known that Cleopatra mandarin, a commercial rootstock, is able to restrict Cl^- transport to the aerial part whereas leaves of Carrizo citrange (another widely used rootstock) becomes rapidly intoxicated in the presence of high concentrations of NaCl (López-Climent *et al.* 2008). In response to different environmental stresses a crucial role for abscisic acid (ABA) and jasmonic acid (JA) has been suggested (Arbona and Gómez-Cadenas 2008).

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Abbreviations: ABA - abscisic acid; CC - Carrizo citrange; CM - Cleopatra mandarin; JA - jasmonic acid; SA - salicylic acid.
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The objective of this work was to elucidate Cd translocation from the roots to the leaves in citrus and changes in phytohormones in response to Cd stress.

One-year-old seedlings of Carrizo citrange (CC; *Poncirus trifoliata* L. Raf. × *Citrus sinensis* L. Osb.) and Cleopatra mandarin (CM; *Citrus reshni* Hort. *ex.* Tan.) were transplanted to 2 dm^3 plastic pots with *Perlite* as a substrate. Plants were allowed to acclimate for one month in a greenhouse with natural photoperiod, 25/18 ºC day/night temperature and 60 - 85 % relative humidity. During this period, plants were watered three times a week with 0.5 dm^3 of a half-strength Hoagland solution (López-Climent *et al.* 2008). Two different experiments were performed. *1*) Three groups of 14 plants per genotype were set as control plants, plants treated with 1.5 mM Cd^{2+} and plants treated with 3 mM Cd^{2+} (Cd-treated plants were watered three times a week with the nutrient solution mentioned above plus $Cd(NO_3)_2$ to achieve the desired Cd^{2+} concentrations and control plants were identically watered but $Cd(NO_3)_2$ was omitted). *2*) Sixty nine seedlings for each genotype were separated in three different groups. One group was set as a control and the other two were treated with $Cd(NO_3)_2$ (30 µM Cd^{2+} and 150 µM Cd^{2+}). Plants were watered as in the first experiment.

In response to Cd treatment, plants showed symptoms of leaf damage such as vein yellowing and curling. Plants showing a percentage of damaged leaves equal or above 50 % were considered cadmium "affected". Leaf gas exchange parameters were measured with a *LCpro+* portable infrared gas analyzer (*ADC Bioscientific,*. Hoddesdon, UK) under ambient CO_2 and humidity (López-Climent *et al.* 2008). After instrument stabilization, measurements were taken on 12 mature leaves. For analyses, frozen plant material was ground to a fine powder using a pre-chilled mortar and pestle. Part of that tissue was stored at -80ºC while other was immediately lyophilized. Cadmium content was determined using 1 g of plant fresh mass that was digested with 10 cm^3 of 35 % nitric acid (*Panreac S.A.*, Barcelona, Spain) for 3 h in an oven at 120 ºC. After that, extracts were filtered and Cd^{+2} content was measured by inductively coupled plasma mass spectrometry (*ICP-MS 7500cx Agilent*, Santa Clara, CA, USA). Plant hormones were assayed according to Arbona and Gómez-Cadenas (2008) by using liquid chromatography linked to a mass spectrometer. All data presented are means ± standard errors. Statistical analyses were performed using *StatGraphics Plus* (*V. 2.1.*) for *Windows* (*Statistical Graphics Corp.*, Warrenton, VA, USA). Differences between treatments were compared by using the least significant difference (LSD) test ($P \leq 0.05$).

In preliminary experiments, citrus plants showed a remarkable tolerance to Cd. Therefore, plants watered with a solution containing 30 µM Cd^{2+} did not show observable leaf damage symptoms for more than three months. In plants treated with 150 or 300 µM Cd^{2+}, first symptoms were observable after 50 d and no plant death occurred throughout a 205-d experimental period. Higher metal concentration were more toxic for CC and, in a few days, all plants watered with 1.5 or 3 mM Cd^{2+} were affected (Table 1).

To test the ability of citrus genotypes to exclude Cd from the aerial part, CC and CM plants were watered for one month with a solution containing toxic concentrations of Cd. Very high endogenous levels of Cd^{2+} were detected in roots of both genotypes when the watering solution was supplemented with either 1.5 mM or 3 mM Cd^{2+} (Table 2). Metal buildup in roots was fast and progressive in plants under both treatments and virtually no differences in this parameter were observed between CC and CM. Cd^{2+} concentration in leaves was markedly lower than in roots in both genotypes (Table 2). Furthermore, Cd accumulation in leaves was distinct between the two genotypes, being CM able to exclude even more Cd from the aerial part than CC.

Leaf Cd concentration was correlated with damage. Although both genotypes suffered from important damage under the high doses of Cd, CM was less affected (Table 1). Leaf damage was also correlated with the inhibition of net photosynthetic rate. Although reduction in net photosynthetic rate was observed in both genotypes, it was higher in CC. Similar trends were observed when the transpiration rate and the stomatal conductance were studied under these high doses of Cd (Table 1).

Table 1. Effect of Cd treatments on leaf damage (means ± SE, $n = 14$) and gas exchange parameters ($n = 12$) in citrus plants CC and CM. *Asterisks* denote significant differences at $P \leq 0.05$ between control and treated plants on each date.

Plant	Cd [mM]	Affected plants [%]		Net photosyntetic rate [µmol m^{-2} s^{-1}]		Transpiration rate [mol m^{-2} s^{-1}]		Stomatal conductance [mmol m^{-2} s^{-1}]	
		10 d	25 d	10 d	25 d	10 d	25 d	10 d	25 d
CC	0	0	0	5.71±0.32	7.26±0.27	1.29±0.06	1.49±0.04	48.05±2.86	66.66±2.44
	1.5	72±1*	85±2*	3.52±0.35*	0.67±0.22*	0.97±0.06*	0.39±0.03*	31.38±2.07*	16.94±1.63*
	3.0	88±2*	100±3*	1.59±0.18*	0.42±0.11*	0.59±0.03*	0.16±0.01*	16.66±1.11*	10.83±2.33*
CM	0	0	0	2.65±0.14	2.43±0.13	0.66±0.12	0.67±0.03	24.44±0.83	25.00±1.16
	1.5	16±2*	60±3*	2.71±0.15	0.35±0.11*	0.73±0.03	0.27±0.02*	28.33±1.93*	11.10±0.10*
	3.0	56±1*	83±2*	1.80±0.13*	0.10±0.02*	0.50±0.03*	0.23±0.01*	18.33±1.16*	10.05±0.06*

Table 2. Effect of Cd treatments on endogenous cadmium and hormone contents in roots (R) and leaves (L) of citrus plants CC and

CM (means ± SE, $n = 3$, *asterisks* denote significant differences at $P \leq 0.05$ between control and treated plants on each date).

Plant	Cd [mM]	Cd^{2+} [µg g^{-1}(F.W.)] 10 d	25 d	JA [ng g^{-1}(F.W.)] 10 d	25 d	ABA [ng g^{-1}(F.W.)] 10 d	25 d	SA [ng g^{-1}(F.W.)] 10 d	25 d
CC-R	0	0.6±0.1	0.7±0.1	113.9±7.1	153.9±2.8	11.8±1.8	4.4±0.6	14.4±3.0	21.8±5.4
	1.5	690±26*	6610±259*	43.4±0.2*	27.7±1.2*	8.5±0.6	5.6±0.8	11.6±1.9	23.5±3.2
	3.0	2219±92*	10924±266*	30.0±5.6*	19.2±4.9*	7.3±0.3*	3.1±0.1*	10.7±0.8	18.8±6.2
CM-R	0	0.9±0.2	0.2±0.1	109.0±10.3	207.2±12.5	5.8±0.1	6.4±0.9	19.2±0.6	12.6±1.0
	1.5	833±37*	7022±13*	93.3±6.8*	71.1±2.7*	5.3±0.8	5.0±0.4	9.8±1.6*	10.5±1.2
	3.0	2274±138*	10552±157*	85.3±12.0*	53.8±4.2*	4.4±0.2*	4.1±0.8	14.2±0.2*	11.8±2.0
CC-L	0	0.010±0.007	0.024±0.001	104.0±17.1	59.1±3.5	94.1±5.8	71.7±7.6	107.8±4.8	75.0±5.3
	1.5	0.106±0.013*	0.358±0.012*	62.4±3.8*	44.8±2.5*	123.1±3.4*	176.8±1.6*	165.9±3.7*	101.2±6.9*
	3.0	0.222±0.073*	0.567±0.067*	198.6±5.9*	104.8±2.3*	287.1±7.7*	144.4±1.1*	275.3±9.7*	122.2±9.0*
CM-L	0	0.014±0.003	0.027±0.006	52.7±7.6	88.6±1.4	56.7±2.1	44.3±10.5	28.3±2.6	51.3±6.1
	1.5	0.074±0.009*	0.103±0.003*	18.3±1.6*	16.1±1.1*	87.1±10.1*	389.1±70.7*	30.6±0.8	99.9±4.2*
	3.0	0.225±0.070*	0.193±0.008*	75.1±0.3*	54.6±0.6*	134.8±9.9*	915.8±13.1*	62.5±2.3*	91.9±9.1*

Very similar pattern of hormone (JA, ABA and SA) changes in response to Cd were found in both genotypes independently of their different tolerance to this metal (Table 2). In roots, a decrease in JA content was observed in both genotypes and at both Cd concentrations while ABA and SA contents were less affected even if a decreasing pattern could be perceived. In leaves, a different picture was observed and ABA and SA contents increased in both genotypes in practically all data points but irregular variations in JA content were observed in response to increased Cd concentrations.

In the second experiment, plants of the two citrus genotypes were watered for 85 d with solutions containing 30 µM or 150 µM Cd^{2+}. Differences in Cd^{2+} uptake between CC and CM persisted under exposure to moderate Cd levels (data not shown). Interestingly, no visible symptoms of damage were observed throughout the studied period. In the same way, gas exchange parameters decreased less dramatically than in the previous experiment (data not shown). Neither roots nor leaves of citrus under these Cd^{2+} treatments showed clear hormonal variations.

From the data presented in this work, it seems clear that citrus genotypes are not Cd hyperaccumulators. Different parameters have been defined to include a species in this category (McGrath and Zhao 2003), including a shoot-to-root ratio of metal concentration greater than one (accounting for an efficient root-to-shoot transport), and a hypertolerance to metals inside cells. Data in Table 2 exclude citrus from this classification because CC and CM plants showed a high Cd retention capacity in the roots even when plants were watered with high doses of Cd^{2+}, endogenous Cd content in leaves was below 0.6 µg g^{-1}, being this level much lower than those reported in different tree species under Cd stress (Moreno-Jiménez *et al.* 2009). Our data are in agreement with previous reports indicating that roots of some woody species have a special capacity for retaining Cd (Unterbrunner *et al.* 2007, Brunner *et al.* 2008).

Despite the well-known sensitivity of citrus to high salinity in the watering solutions or drought (Gómez-Cadenas *et al.* 1996, López-Climent *et al.* 2008), data presented in this work indicate that citrus plants tolerate Cd^{2+} to a certain extent. When watered with solutions containing 30 or 150 µM Cd^{2+}, citrus did not show visible damage for more than two months. Interestingly, the extremely low Cd^{2+} concentrations found in leaves in comparison to the roots (Table 2) seem to support the hypothesis that most of the aerial damage observed (Table 1) is due to a root malfunctioning. A similar situation was observed in citrus plants under continuous soil flooding (Arbona and Gómez-Cadenas 2008).

The lower Cd translocation to the leaves found in CM than in CC could be related, at least in part, to the lower transpiration of this genotype (Table 1). It should be noted that recent research demonstrates that different processes are involved in the Cd transport from roots to shoots. These key processes would include immobilization inside the vacuoles of root cells, metal precipitation or binding of free metal cations to cell walls that can also slow down their movement into the shoot with the transpiration stream. Recently metal pumps responsible for the loading of zinc ions into the xylem have been proposed as essential in the accumulation of Cd into the shoot (Wong *et al.* 2009). In our system, all these factors are probably playing a key role in the translocation to the shoots but due to the high levels of Cd entering in the roots it could be also a plausible explanation that different transpiration rates have an influence in Cd translocation to the leaves. Other authors have linked transpiration to Cd buildup in aerial parts in different species (Salt *et al.* 1995, Van der Vliet *et al.* 2007). Even more, López-Climent *et al.* (2008) showed that in citrus chloride absorption and hence salt tolerance was linked to water use. In those studies, CM having a lower transpiration rate accumulated less chloride ions in leaves than CC.

As raised by Clemens (2006), it is still a non-

answered question whether plant responses are specific to Cd toxicity while this element apparently has no biological function. Citrus roots under different concentrations of Cd, either did not respond or reduce ABA, SA and JA contents independently of the different tolerance to Cd observed in the two studied genotypes. This fact clearly indicates that there is no a specific hormonal response to the metal buildup in citrus genotypes. The so-called "stress hormones" did not clearly respond to this stimulus while it has been shown that the same genotypes are able to accumulate large amounts of phytohormones in roots under water stress (Gómez-Cadenas *et al.* 1996). The relative decreases in root JA and ABA levels had been previously observed (Arbona and Gómez-Cadenas 2008) in citrus under continuous flooding conditions and could reflect a progressive decay of root activity. Contrastingly, those plants under high toxic conditions showed progressive increases in leaf concentration of the stress hormones. These signaling events must be an indirect effect derived from a root malfunctioning (Xu *et al.* 2009) and therefore a general response to stress. Due to the extraordinarily low accumulation of Cd in leaves (in comparison with roots), it seems unlikely that the observed accumulation of hormones could be a specific response to the metal. Moreover, no important variations in hormonal profiles in leaves of the same plants were observed when Cd excess was not toxic for plants. All these data together seem to discard that endogenous JA, ABA or SA may function in citrus as specific signaling molecules that sense Cd toxicity.

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