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Study on metal-triggered callose deposition in roots of maize and soybean*

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Abstract: Callose plays important roles in a variety of processes of plant development, and/or in a response to a range of biotic and abiotic stresses. In the current work we have studied and compared the effect of lead, cadmium and arsenic on accumulation of newly formed callose deposits in the roots of maize and soybean. We observed formation of characteristic callose deposits in the root cell walls, probably associated with plasmodesmata, depending on the type of metal and the plant species investigated. Further, the callose turnover was analysed by measuring of total callose content as well as activities of total β-(1,3)-glucanases in roots. The latter enzymes are responsible for callose depletion, and their possible role during metal stress has previously been proposed. However, neither of these biochemical values appeared to be sufficiently reliable for scoring the altered callose turnover (including local deposits) in plant tissue. The microscopical observations are discussed in light of the biochemical data obtained.

Key words: β-(1,3)-glucanases; callose; metal stress; PR-2.

Abbreviations: As, arsenic; cc, central cylinder; Cd, cadmium; co, cortex; en, endodermis; ep, epidermis; FW, fresh weight; Pb, lead; ph, phloem; PR, pathogenesis-related protein; TI, tolerance index; x, xylem.

Introduction

Callose is a β-(1,3)-D-glucan that occurs widely as a cell wall component in the course of plant development and in specialized reproductive tissues at particular stages of differentiation. For example, it is an important part of the septum (the phragmoplast) arising during cell division (Brown & Lemmon 2009), sieve plates in dormant phloem (Stone & Clarke 1992), growing pollen tubes (Rae et al. 1985; Newbigin et al. 2009), seed hairs (Huwyler et al. 1978; Maltby et al. 1979), and is also a structural component of plasmodesmata (Lucas et al. 1993; Turner et al. 1994; Rinne et al. 2001; Levy & Epel 2009). Furthermore, callose is deposited between the plasma membrane and the cell wall after exposure of plants to a range of abiotic and biotic stresses, e.g., wounding, desiccation, metal toxicity or microbial attack (Stone & Clarke 1992; Donofrio & Delaney 2001; Verma & Hong 2001; Kartusch 2003; Hirano & Brunner 2006; Hrmova & Fincher 2009; Stone 2009).

The role of callose in cell wall biology has been argued for a long time as its accumulation is usually only transitory (Zabotin et al. 2002; Hrmova & Fincher 2009). It has been suggested to serve specific biological functions such as a scaffold or matrix for the addition of other cell wall components (Brown & Lemmon 2009), as a permeability barrier participating in the inter-cellular communication (Levy et al. 2007; Zavaliev et al. 2011), or as a thickening substance (Stone & Clarke 1992; Brown & Lemmon 2009). More recent results suggest, however, that its function may be even more complex (Jacobs et al. 2003; Nishimura et al. 2003). Changes in the deposition of callose mirror the output of balance between its synthesis and degradation. Callose is synthesized at membranes as it has never been observed in Golgi stacks or vesicles (Brown & Lemmon 2009). Its synthesis is mediated by a multi-subunit enzyme complex β-(1,3)-glucan synthase that is located on the cytoplasmic surface of the plasma membrane (Stone & Clarke 1992; Verma & Hong 2001; Brown & Lemmon 2009). The cytoplasmic membrane has a constant level of an inactive subunit of this enzyme complex – the callose synthase, which under conditions of stress gets activated quickly (Chen & Kim 2009). On the other hand, callose is degraded by specific β-(1,3)-glucanases (endohydrolases) (Zabotin et al. 2002; Bacic et al. 2009) that belong to a group of enzyme proteins related particularly to plant pathogenesis (pathogenesis-related pro-

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teins; PR-2) (van Loon & van Strien, 1999). Unfortunately, the depletion of callose by β-(1,3)-glucanases is much less studied than its synthesis (Iglesias & Meins 2000; Ruan et al. 2004).

The mechanism of callose accumulation is controlled by distinct signalling pathways, depending (at least partly) on the environmental conditions (Luna et al. 2011). For example, a rapid synthesis and deposition of callose is a common response of plant host cells following microbial attack (Hrmova & Fincher 2009). On the other hand, decomposition of callose can already occur within 5–10 minutes after tissue injury (Nakashima et al. 2003). Plants (including algae) react by increased accumulation of callose also to excess of metals (Bacic et al. 2009; Krzesłowska 2011); its rapid deposition in roots upon exposure to aluminium (observable already after less than 1 h) is considered as the most sensitive indicator of this type of stress (Horst et al. 1997; Sivaguru et al. 2000; Qin et al. 2007; Stass et al. 2007). Formation of characteristic callose deposits was also observed as a general response of plants to many other metals. Exposure to excess of metals, e.g., lead, manganese, has led to variable callose deposition (Peterson & Rauser 1979; Wissemeier et al. 1992; Fecht-Christophers et al. 2002; Krzeslowska et al. 2010).

The ability of plant to induce the callose accumulation varies depending on the type and concentration of the applied metal and on the plant species (Wissemeier et al. 1992; Collet et al. 2002) therefore the data reported are not always consistent.

This work was devoted to study the deposition of callose in the roots of the monocot maize and the dicot soybean following exposure to relatively high concentrations of three different metals (lead, cadmium or arsenic) within a single experiment. The microscopical observations were correlated with the measurements of the total callose content in the stressed root tissues, as well as with the data on total β-(1,3)-glucanase activities pointing to callose depletion.

Material and methods

Plant material and growth conditions

Seeds of maize (*Zea mays* cv. Quintal) and soybean (*Glycine max* cv. Korada) were surface-sterilized with 5% (v/v) sodium hypochloride for 5 min and then rinsed five times in distilled water at room temperature. The seeds were cultivated in Petri dishes (ø 15 cm) lined with two layers of water-moistened filter papers (Whatman No.1) in the dark at 25 °C until the roots reached 3–8 mm in length. The uniformly germinated seeds were then transferred on the fresh filter papers moistened with distilled water (control) or various concentrations of lead (500 mg L^{-1} $Pb(NO_3)_2$), cadmium (300 mg L^{-1} $Cd(NO_3)_2.4H_2O$) or arsenic (100 mg L^{-1} As_2O_3). Cultivation continued for 48 hours in the dark at 25 °C as reported earlier (Békésiová et al. 2008; Piršelová et al. 2011). The fresh weight (FW) of roots was determined gravimetrically.

Histochemical detection of callose in root tissues

The root segments (~5 mm) of control and metal-stressed seedlings were fixed in Navashin's solution for 24 hours, dehydrated and embedded in a paraffin wax according to standard procedures. The sections (7 µm in thickness) were cut 2–3 mm apart from root apex with a MicroTec Cut 4055 rotary microtome and examined for their autofluorescence using a Zeiss Axioplan 2 microscope equipped with a Zeiss filter set 01 (exciter BP 365 nm, beam splitter FT 395 nm, and barrier LP 397 nm). Subsequently they were stained with 0.1% (w/v) Aniline Blue (Serva) in 0.5 M potassiumphoshate buffer (pH 8.0) for 10 min as described previously by Stone & Clarke (1992), washed briefly in distilled water and examined again with the same filter set. Images were recorded using a Sony DXC-S500 color digital camera system and Axiovision software version 4.8 (Zeiss).

Callose content measurement

Callose content in ~1 cm long root tips was assayed using 0.1% (w/v) aqueous Aniline Blue (Serva, Heidelberg, Germany) according to the protocol of Hirano & Brunner (2006). Its concentration was quantified with a Titertek FLUOROSCAN II fluorimeter at excitation and emission wavelengths of 400 and 490 nm, respectively. All extracts were assayed at least five times in triplicate per flask and each extract. Pachyman was used as a reference and the callose concentration was expressed as µg of pachyman equivalents per mg root fresh weight (µg PA mg^{-1}FW). For each root sample, the fluorescence intensities in the absence of the stain (the autofluorescence) were subtracted from the intensities in the presence of the stain.

β-1,3-glucanase assay

The activity of total β-(1,3)-glucanases was assayed fluorimetrically in a crude protein extracts (Hurkman & Tanaka 1986) from both control and metal-stressed roots of maize and soybean as described previously by Siefert & Grossmann (1997) with the same fluorimeter and excitation/emission wavelengths as for the callose content measurement. All extracts were assayed at least five times in triplicate per flask and each extract. Here, laminarin was used as the substrate and the enzyme activity was expressed in katal mg^{-1} cell protein, based on the release of 1 mol s^{-1} D-glucose from laminarin. The activity of β-(1,3)-glucanases in metal-stressed samples was expressed with respect to the activities of the corresponding controls.

Statistical analyses

Statistical significance was evaluated by Student's t test using Microsoft Excel for Windows XP.

Results

The effect of metal stress on the root growth

The response of maize and soybean roots to 48 hours exposure to lead (500 mg L^{-1}), cadmium (300 mg L^{-1}) or arsenic (100 mg L^{-1}), respectively are presented in Table 1. The measurements of fresh weight of both control and metal-stressed roots showed that the given doses of metals caused significant inhibition of the root growth, while arsenic exerted strongest (decrease to 23.81% of FW for maize, and to 35.71% for soybean) and lead the least (decrease to 61.90% of FW for maize, and to 85.71% for soybean) toxicity for both the plant species.

Table 1. The effect of different concentrations of lead (Pb), cadmium (Cd) or arsenic (As) on the fresh weight of roots of maize and soybean with respect to water-treated control.

Metal (mg L^{-1})	Maize	Soybean
	FW of roots (g)	FW of roots (g)
Control	0.21 ± 0.01	0.14 ± 0.01
Pb 500	0.13 ± 0.02 ***	0.12 ± 0.01 *
Cd 300	0.06 ± 0.00 ***	0.08 ± 0.01 ***
As 100	0.05 ± 0.01 ***	0.05 ± 0.00 ***

Data indicate ± standard error of mean values of 30–100 roots. The data from analyses significant at $P < 0.05$ and $P < 0.001$ are marked with * and ***, respectively.

Callose detection in situ

A faint autofluorescence was visible in the un-stained sections of both control and metal-stressed roots of maize and soybean; exception was the strong autofluorescence of the xylem vessels in both types of the tissue and the suberinized cell walls of the endodermis in the maize tissue. After staining with the Aniline Blue dye, callose was clearly localized in the phloem (the sieve elements) in the control roots of both species (Figs 1A–B and 2B, arrowheads) as well as in the metal-stressed roots of soybean (Figs 1D–E, G, J, K, arrowheads).

When compared with roots of control soybean, we further observed the presence of numerous multiple or

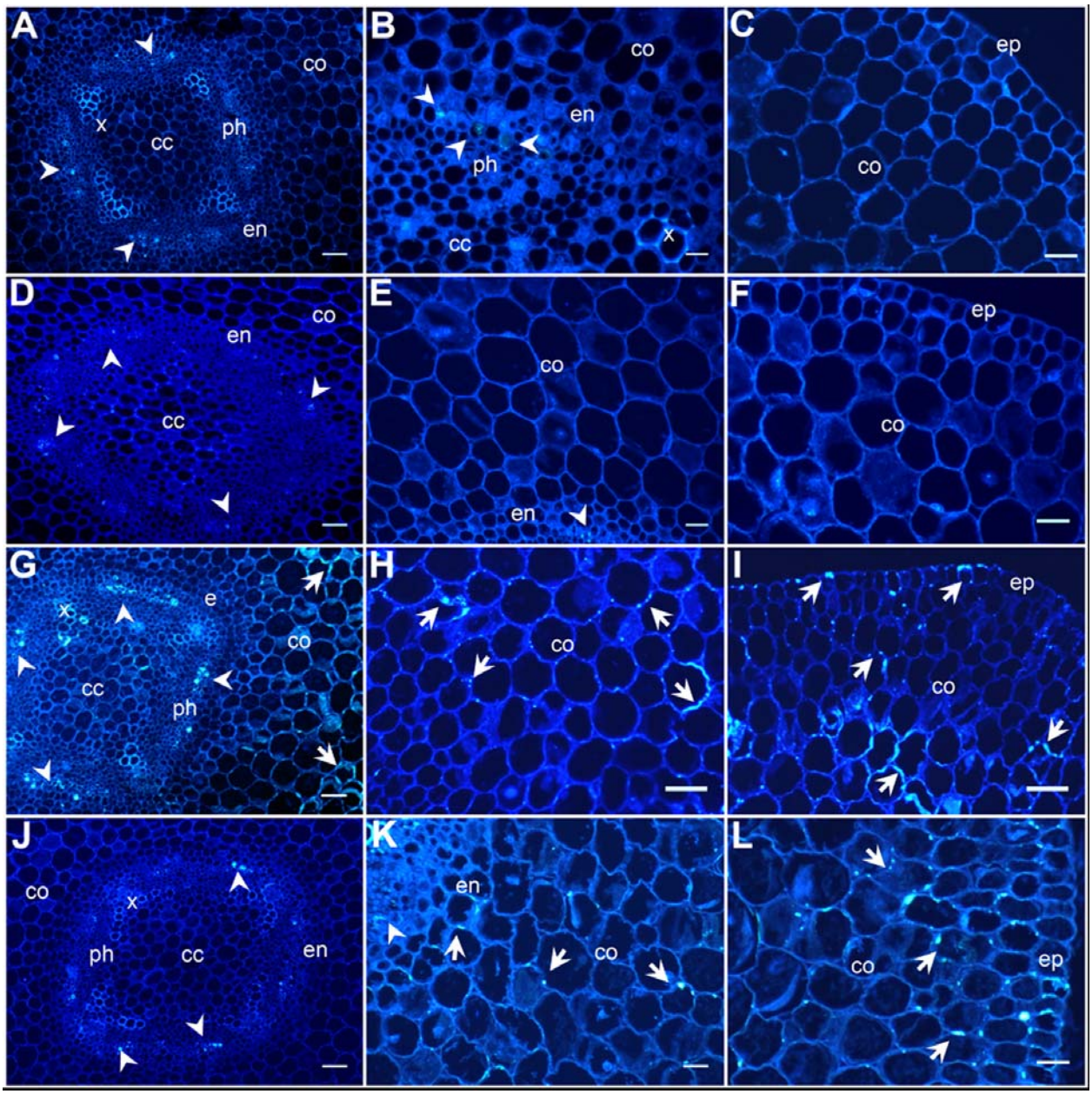

Fig. 1. The pattern of callose deposition in the root tissue of soybean exposed to lead (D–F), cadmium (G–I) or arsenic (J–L) with respect to corresponding (water-treated) control (A–C). Micrographs of paraffine-embedded and cross-sectioned primary roots stained with the Aniline Blue dye (at pH 8). Light-blue spots indicate aggregates of newly formed callose deposits (arrows) on the faint blue autofluorescent background in the central cylinder (first column), cortex (middle column); epidermis (last column). Callose typical deposition in the sieve elements of the phloem is marked with arrowheads. Abbreviations: cc: central cylinder; co: cortex; en: endodermis; ep, epidermis; ph: phloem; x: xylem. Scale bars = 100 μm.

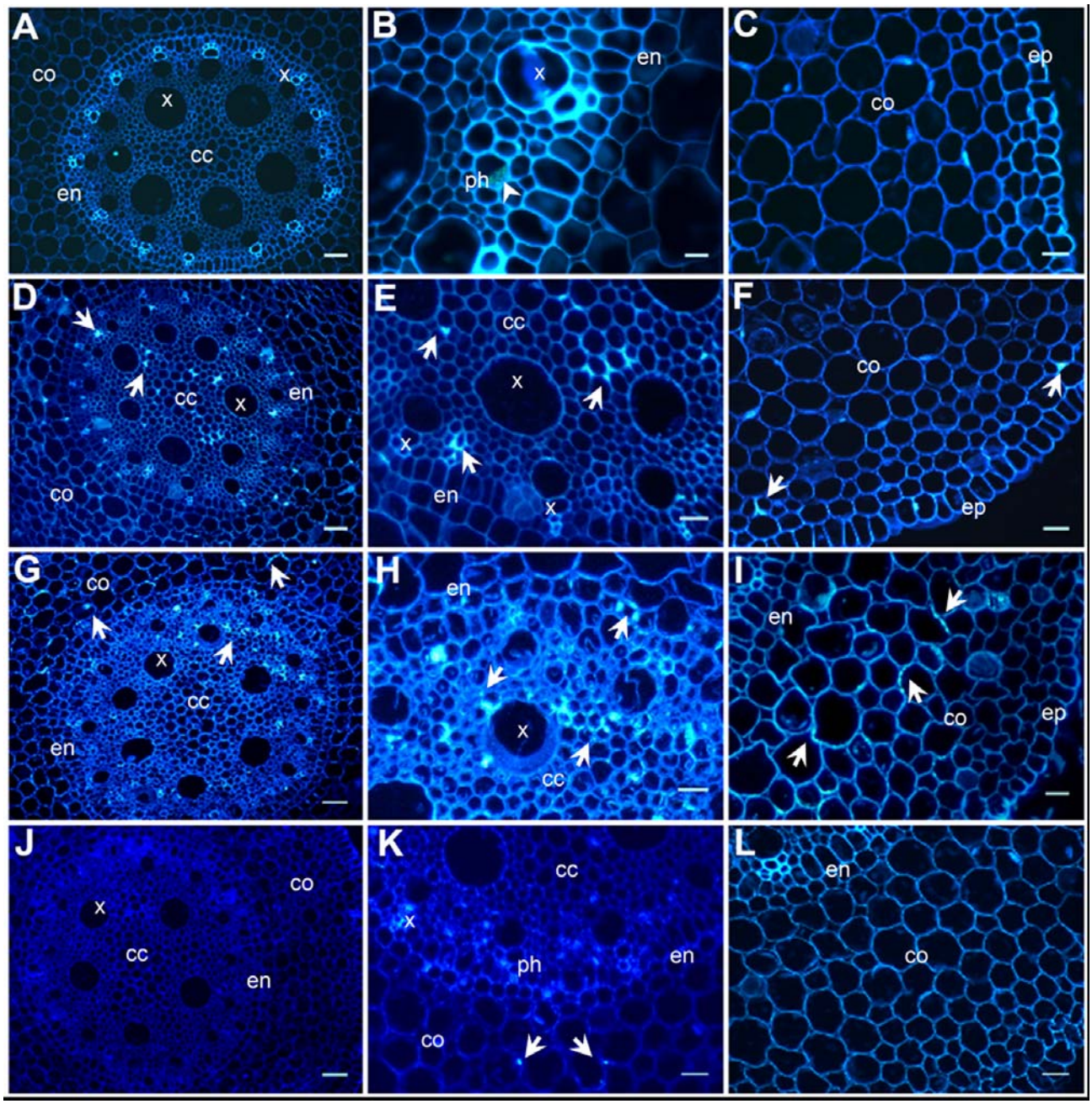

Fig. 2. The pattern of callose deposition in the root tissue of maize exposed to lead (D–F), cadmium (G–I) or arsenic (J–L) with respect to corresponding (water-treated) control (A–C). Micrographs of paraffine-embedded and cross-sectioned maize roots stained with the Aniline blue dye (at pH 8). Light-blue spots indicate aggregates of newly formed callose deposits (arrows) on the faint blue autofluorescent background in the central cylinder (first column), cortex (middle column); epidermis (last column). Callose typical deposition in the sieve elements of the phloem (B) is marked with arrowheads. Abbreviations: cc: central cylinder; co: cortex; en: endodermis; ep, epidermis; ph: phloem; x: xylem. Scale bar = 50 μm in B, 100 μm in A, C–L.

single knoblike callose deposits in the walls of the cells in the epidermis, cortex and endodermis (arrows) in the root tissues treated with cadmium (Figs 1G–I, arrows) or arsenic (Figs 1J–L, arrows). The newly formed callose deposits were sporadically located in the walls of the cells of the central cylinder as well (Fig. 1G, arrows). In some parenchyma cells of the cortex, callose was clearly deposited throughout the cell walls of cadmium-stressed roots (Figs 1G and I). In contrast, the lead treatment did not result in any additional callose deposits in the soybean root tissue (Figs 1D–F).

In roots of maize treated with cadmium, we observed the deposition of callose in the cell walls of the cells in the central cylinder (Figs 2G–H, arrows), epidermis and cortex (Fig. 2I, arrows). On the other hand, in the roots stressed with lead only single knoblike callose deposits were sporadically observed in the cell walls of the cells in the epidermis, cortex and central cylinder (Figs 2D–F, arrows). Similarly, following the arsenic stress, only rare (even completely absent) local accumulations of callose were detected in the maize roots (Fig. 2K, arrows).

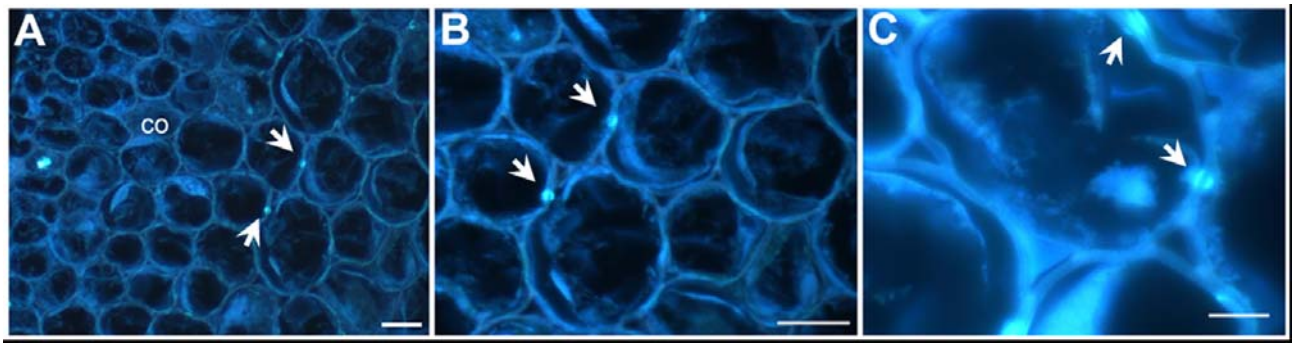

Fig. 3. Callose deposition in the root tissue of soybean exposed to arsenic as revealed by Aniline Blue staining at different magnifications (A–C). Light-blue spots indicate aggregates of newly formed callose deposits (arrows) in the walls of the cells of the cortex (co), on the faint blue autofluorescent background. The presence of callose in the cell walls is probably associated with plasmodesmata. Scale bar = 50 μm in A and B, 20 μm in C.

Table 2. Relative total callose content in roots of maize and soybean exposed to different concentrations of lead (Pb), cadmium (Cd) or arsenic (As) with respect to water-treated control.

Metal (mg L^{-1})	Maize	Soybean
Control 0	1.00 ± 0.00	1.00 ± 0.00
Pb 500	1.45 ± 0.19 *	1.34 ± 0.20
Cd 300	1.21 ± 0.06 **	1.10 ± 0.08
As 100	1.05 ± 0.10	0.90 ± 0.14

Data indicate ± standard error of mean values of five replicates. The data from analyses significant at $P < 0.05$ and $P < 0.01$ are marked with * and **, respectively.

Table 3. Relative total activity of β-(1,3)-glucanases in the protein extracts from the roots of maize and soybean exposed to different concentrations of lead (Pb), cadmium (Cd) and arsenic (As) with respect to water-treated control.

Metal (mg L^{-1})	Maize	Soybean
Control 0	1.00 ± 0.00	1.00 ± 0.00
Pb 500	0.97 ± 0.05	1.47 ± 0.06 **
Cd 300	0.95 ± 0.03	1.06 ± 0.01 *
As 100	0.80 ± 0.02	0.66 ± 0.07 *

Data indicate ± standard error of mean values of 5–6 replicates. The data from analyses significant at $P < 0.05$ and $P < 0.01$ are marked with * and **, respectively.

In the metal-stressed roots, the presence of callose in the walls of the cells in the epidermis and cortex may be associated with plasmodesmata (Fig. 3)

Callose content in roots

Total callose content was quantified fluorimetrically in both control and metal-stressed roots of maize and soybean (Table 2). The obtained values related to corresponding controls showed that the total content of callose increased (though not significantly) in soybean roots exposed to both lead (by 33.85%) and cadmium (by 16.33%). In contrast, no change was detected in roots exposed to arsenic. Alterations in the callose content in the roots of maize were similar; elevated callose content was observed due to effect of lead (by 57.05%, $P < 0.05$) and cadmium (by 20.76%, at $P \leq 0.01$), while the total callose content remained almost unaltered in the maize roots stressed with arsenic (Table 2).

Activity of β-(1,3)-glucanases in roots

The activity of total β-(1,3)-glucanases has been measured in crude protein extracts from roots in order to put the data into context with the metal-triggered changes of the callose content. Compared to control (Table 3), the activity of β-(1,3)-glucanases in soybean was induced significantly after treatment with lead (at $P < 0.01$) and cadmium ($P < 0.05$), but inhibited upon stress with arsenic ($P < 0.05$). In the maize roots, surprisingly, the activity of β-(1,3)-glucanases remained almost unaltered (non-significantly inhibited) irrespective on the type of the metal applied (Table 3).

Discussion

The β-(1,3)-D-glucans are widely distributed in plants, algae, fungi, euglenoid protozoas and bacteria, where they are involved in cell wall structure and in a range of other biological functions (Hrmova & Fincher 2009). These polysaccharides are commonly referred to as callose in higher plants. The precise role of callose is still not known, but it is commonly employed in a local thickening of plant cell walls during the plant development (Stone & Clarke 1992; Chen & Kim 2009). Callose probably serves as a scaffold or a matrix onto- or into which the more permanent polysaccharides and proteins of the mature wall may be deposited (Stone & Clarke 1992; Brown & Lemmon 2009). Most likely, callose is used to transiently stabilize the membranes when it is necessary (Brown & Lemmon 2009). It has been suggested that callose may function as a "molecular sieve" enabling the plant (or plant cell) to regulate the intake (uptake) of minerals (including toxic metals) (Krzeslowska 2010). At the same time, it may act as a "physiological sealant" and prevent a leakage of assimilates (or other nutrients), as well as it can cause inhibition of cell-to-cell trafficking (Sjölund 1997; Chen & Kim 2009) in the stress conditions.

In this study we aimed to detect possible alterations in the deposition/accumulation of callose in the

roots of maize and soybean following stress by three different types of metals. We observed light-blue fluorescent spots indicating newly formed deposits of callose in the metal-stressed roots after Aniline Blue staining (Stone et al. 1992). However, the number, size, location and distribution of the callose deposits was variable in relation to the metal stress applied and the plant species (Figs 1D–L and 2D–L) when compared to roots of control plants (Figs 1A–C and 2A–C). Most obviously the callose deposits were densely scattered in the root tissues exposed to cadmium in both soybean and maize (Figs 1G–I and 2G–I, respectively). Upon exposure to arsenic, high number of callose deposits was present in soybean (Figs. 1J–L). In contrast, only few newly formed callose deposits were observed in arsenic-stressed roots of maize (Fig. 2K). Regarding the stress with lead, callose deposits were rare (in maize, Figs 2D–F) or even absent (in soybean, Figs 1D–F), most probably as a consequence of a relatively low level of metal toxicity (Table 1). Previously, lead-induced callose deposition has been detected in the rhizodermis and in the centre of the stele of lead-treated soybean root tips (Samardakiewicz et al. 1996).

In the metal-stressed roots, the presence of callose in the walls of the cells in the epidermis and cortex may be associated with plasmodesmata (Fig. 3) as was suggested previously (e.g., Sivaguru et al. 2001; de Cnodder et al. 2005). Plasmodesmata serve as membranous channels with a high degree of plasticity allowing direct cytoplasmic cell-to-cell communication of both small molecules and macromolecules (Levy & Epel 2009). A range of stressors can transiently change their conductivity and influence a transfer rate of nutrients (Turner et al. 1994; Rinne et al. 2001; Ruan et al. 2004; Levy & Epel 2009). The presence of a higher amount of callose in the cell walls surrounding the plasmodesmata has been shown to result in their closure and subsequent inhibition of root growth (Turner et al. 1994; Sivaguru et al. 2000; Rinne et al. 2001; Levy & Epel 2009).

The more pronounced accumulation of callosic material in soybean (Fig. 1) compared to maize (Fig. 2) contradicts the assumption that metal-triggered synthesis and appearance of this polysaccharide is more characteristic for sensitive lines (reviewed in Poschenrieder et al. 2008). The plant defense potential, however, can depend on many factors, while in the case of the two plant species investigated the composition of the cell wall (including callose) might be of importance. Mono- and di-cotyledonous plants greatly differ in their cell wall structure (Henry & Harris 1997), impacting permeability, metal-binding capacity, and consequently metal tolerance (Corrales et al. 2008).

We further performed measurements of total callose content (Table 2) in roots in order to complement our microscopic observations with total activity of β-(1,3)-glucanases (Table 3) and identify possible coincidence (Piršelová et al. 2011). As reported previously (Zabotin et al. 2002) the activity of β-(1,3)-glucanases and the rate of callose depletion correlate, and common mechanisms control the activities of endoglucanase(s) and callose synthase(s). Although we did not expect a perfect match between these parameters, we looked for their indicative value in a context of our (microscopical) study. Indeed, we found coincidences between the increased total callose content (Table 2), decrease of glucanases enzyme activities (Table 3) as well as with the appearance of newly formed callose deposits in both soybean and maize roots exposed to cadmium stress (Figs. 1G–I and 2G–I, respectively). However, in the cases of arsenic and lead stress, the data on the three parameters considered did follow the proposed scheme inconsistently or even were contradictory. For example, despite of a significant increase of total callose content in the roots of lead-stressed maize (Table 2) and lead-stressed soybean (Table 2), there were no newly formed deposits visible *in situ* in the given part of the root (Figs. 2D–F and 1D–F, respectively), while the total glucanase activities were reduced and induced in the two species, respectively (Table 3).

There might be several explanations for the observed phenomenon. First, the deposition/depletion of callose is a local, very flexible process, while the occurring changes might not readily be pronounced at the level of total callose content. In addition, the widely applied method of Hirano & Brunner (2006) for the measurement of total callose content does posses limitations due to certain level of (possibly still un-neglectable) background. This can be due to polyphenols and different polysaccharides, some of which, e.g., tannins or lignins, reveal metal stress-dependent accumulation (e.g., Chin et al. 2009; Piršelová et al. 2011) that also might contribute to error of the measurements. Second, the value of total glucanase activities also reflects the sum of activities of several isoforms that can be regulated differently (opposingly) under stress. As we have shown previously (Békésiová et al. 2008; Piršelová et al. 2011), it is possible to observe both metal-triggered induction as well as inhibition of certain (glucanase) enzyme isoforms within the same plant protein extract. Both responses can represent an efficient plant strategy against (metal) stress either as an induced defense component, or as a suppressed callose "decomposer" that renders the cell wall less permeable. Nevertheless, the exact role of plant glucanases under metal stress is still unclear.

In conclusion, we showed that upon metal exposure callose accumulate in root tissue, probably in the cell wall surrounding plasmodesmata. The occurrence of deposits, however, was dependent on both the metal type and the plant species. Callose has clearly protective role for the cells, however there are still uncertainties regarding the exact role during (heavy) metal stress. Its deposition is likely to result in reduced cell wall trafficking and growth inhibition that might represent a part of the plant defense strategy against toxic metals. Apparently, neither the values of total callose content nor of total glucanase activities might be sufficiently reliable for scoring the altered callose turnover (including local deposits) in plant tissue. Therefore, *in situ* observations or molecular approaches focusing

on (co)regulation/expression of responsible callose synthase and glucanase genes should additionally be performed to avoid generalization and make relevant conclusions.

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