

# Molecular Mechanisms Involved in Lead Uptake, Toxicity and Detoxification in Higher Plants

B. Pourrut, M. Shahid, F. Douay, C. Dumat and E. Pinelli

**Abstract** Lead (Pb) is one of the most useful metals, and has contaminated most of the ecosystems. It has been known to influence various morphological, physiological and biochemical processes in plants. Higher plants have evolved sophisticated internal detoxification mechanisms, to deal with metal toxicity, that include selective metal uptake, excretion, chelation by specific ligands and compartmentalization. Despite these detoxification systems, Pb is able to induce the production of reactive oxygen species (ROS) which can cause harmful effects on vital constituents of plant cells: protein oxidation, lipid peroxidation, enzyme inactivation or DNA damages. Unlike other metals, little is known about the mechanisms involved in Pb uptake, toxicity, oxidative stress generation and detoxification in plants. This chapter reviews the recent understanding of physiological and biochemical mechanisms involved in Pb uptake and translocation into plants. It also provides a broad overview of the most important mechanisms of Pb toxicity and tolerance in plants.

**Keywords** Genotoxicity · Lead · NADPH oxidase · Pb · Phytochelatins · Oxidative stress

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## Contents

1	Introduction.....	122
2	Plant Absorption of Lead.....	123
3	Lead in the Root and Its Translocation to the Aerial Parts .....	124
	3.1 Radial Diffusion in the Root.....	124
	3.2 Detoxification Mechanisms.....	125
	3.3 Translocation to the Aerial Parts of the Plant.....	127
4	Lead Effects on Plants .....	128
	4.1 Effects on the Cell Constituents .....	129
	4.2 Water Status.....	132
	4.3 Mineral Nutrition.....	133
	4.4 Photosynthesis.....	133
	4.5 Respiration .....	135
5	Lead and Oxidative Stress .....	136
	5.1 Lead-Induced Oxidative Stress.....	136
	5.2 Role of NADPH-Oxidase in Lead-Induced Toxicity.....	136
6	Conclusion and Perspectives.....	140
	References.....	140

## 1 Introduction

Lead (Pb) is one of the most abundant metals polluting the environment. It originates from natural sources but its widespread anthropogenic use has caused global contamination of biotopes and biocoenosis. Apart from certain areas in which atmospheric fallout is significant (Uzu et al. 2009; Uzu et al. 2010; Schreck et al. 2012), Pb mostly penetrates the plant through the roots. Its phytoavailability in soil is largely controlled by complex interactions governed by many biogeochemical factors including pH, redox conditions, mineralogy, biological and microbial conditions, organic and inorganic ligand levels, and competing cation levels (Pourrut et al. 2011b; Shahid et al. 2012b). Once it has penetrated the root system, the majority of Pb remains there. Although there are certain species that are capable of transferring large quantities of Pb to their shoots, the root acts as a very effective barrier against the movement of Pb (Gupta et al. 2013).

Extensive research has found that Pb causes toxic effects in plants. Pb has been described to influence various morphological, physiological and biochemical processes (Seregin and Ivanov 2001; Pourrut et al. 2011b). However, despite numerous studies on Pb toxicity and unlike other metals, only limited data is available on molecular mechanisms involved in Pb uptake, toxicity and detoxification in higher plants. This chapter represents a comprehensive understanding of these mechanisms. Special emphasis will be given to Pb-induced reactive oxygen species (ROS) generation and the central role played by the NADPH-oxidase.

## 2 Plant Absorption of Lead

The upper layers of the radicular cortex (rhizoderm and collenchyma/parenchyma) constitute a physical barrier against Pb penetration into the root. It has been found that initially, Pb present in the soil solution is adsorbed on root surface. It may bind to the carboxylic groups of uronic acids which composed the root mucilage (Glinski and Lipiec 1990) or directly to the polysaccharides of rhizodermic cell surface. Plaque formation of iron oxides and hydroxides on the root surface could limit plant absorption thanks to their capacity to adsorb large quantities of metals (Otte et al. 1987). However, in the case of Pb, these plaques seem to play a less important physical barrier (Ye et al. 1998).

Once it has fixed to the rhizoderm, Pb could penetrate the root system passively and follows the water conduction system. Such absorption is not uniform throughout the root, since a Pb concentration gradient is observed in the tissues, starting from the apex, which is the area of highest concentration (Tung and Temple 1996b; Seregin et al. 2004). The young tissues, and the apical area in particular (excluding the root cap) in which the cell walls are still thin, are the parts of the plant that absorb the most Pb (Wierzbicka 1987). This apical region also corresponds to the area in which the rhizospheric pH is more acidic. This low pH favours metal solubility and a locally high Pb concentration in the soil solution.

At the molecular level, the mechanisms, through which the metal manages to penetrate the roots, have not yet been explained. Pb may benefit from the non-selectivity of some channels/transporters and the very high potential in membrane that can exceed  $-200$  mV in the rhizoderm cells. Pb absorption is therefore a passive absorption, but requires the cell to expend energy in order to maintain this very negative potential through the excretion of protons into the external environment via  $H^+$ /ATPase pumps. In Pb-exposed wheat, the use of vanadate, an inhibitor of these pumps, strongly limited Pb absorption (Wang et al. 2007). Among non-selective cation channels, depolarization-activated calcium channels (DACC), hyperpolarization activated calcium channels (HACC) and voltage-insensitive cation channels (VICC) are thought to be one of the principal routes of Pb entry into root cells (White 2012).

The inhibition of root uptake by the addition of  $Ca^{2+}$ ,  $Mg^{2+}$  or  $K^+$  ions into the external environment is a well-known phenomenon (Kim et al. 2002; Pourrut et al. 2008). The use of calcium channel inhibitors (lanthanum chloride) or those of potassium (caesium chloride) showed that these channels are pathways for root uptake (Wang et al. 2007; Pourrut et al. 2008).

The use of transgenic plants highlighted a family of transmembrane proteins known as HvCBT1 in barley (Schuurink et al. 1998), AtCNGC1 to AtCNGC6 (cyclic nucleotide-gated ion channel) in *Arabidopsis thaliana* (Kohler et al. 1999; Leng et al. 1999) and NtCBP4 in tobacco (Arazi et al. 1999), as one of the non-selective entry pathways used by Pb. This transgenic approach also showed that a Low Affinity Cation Transporter (TaLCT1) could modulate the absorption of  $K^+$ ,  $Na^+$ ,  $Ca^{2+}$  and  $Cd^{2+}$  and would be an alternative entry pathway for  $Pb^{2+}$  in tobacco

roots (Wojas et al. 2007). Recently, Das et al. (2012) showed an up-regulation of a Yellow Stripe-like protein gene (BjYSL 2.8) in Pb-exposed *Brassica juncea* plants.

Other transporters, such as the families of Cation Diffusion Facilitator (CDF), ZRT/IRT-like Protein (ZIP) or the Natural resistance-associated macrophage proteins (Nramps), associated with the transport of copper, zinc, cadmium and manganese (see, for review, Hall and Williams 2003), could also play a role in Pb transport.

Finally, Krzesłowska et al. (2010) hypothesized that Pb could enter the cell protoplast endomembrane system as a pectin—Pb complex during internalization of low-methylesterified pectins from the cell wall.

### 3 Lead in the Root and Its Translocation to the Aerial Parts

#### 3.1 Radial Diffusion in the Root

##### 3.1.1 Apoplastic Pathway

Many histological studies have shown that Pb is essentially transported in the apoplast and that it follows water movements within the plant (Lane and Martin 1977). Once inside the apoplast, Pb can migrate relatively quickly. In onion roots, Wierzbicka (1987) has shown that Pb is transferred at the speed of a layer of cells in 5 min. In maize, however, Seregin and Ivanov (1997) noted restricted diffusion to the epidermis and the upper layers of the cortex, after 24 h of Pb exposure. To a lesser extent, after 48 h of exposure, the conductive tissues were also affected.

Although Pb can diffuse within the root, only a small fraction present in the root is mobile. More than 90 % of Pb is found in insoluble forms (Ernst 1998; Wierzbicka et al. 2007) and is strongly linked to external components of the cells. Pb is mainly linked to the cell walls (Tung and Temple 1996b; Wierzbicka 1998; Jiang and Liu 2010), but can be found associated with the middle lamella (Jarvis and Leung 2001) or the plasma membrane (Seregin et al. 2002; Seregin et al. 2004). It may also be precipitated in the intercellular space (Jarvis and Leung 2001).

This distribution that is very specific to Pb, can be explained by its particular affinity for the carboxyl groups (Inoue et al. 2013) and pectins (Krzesłowska 2011), and, to a lesser extent, to hemicellulose and cellulose molecules (Krzesłowska 2011) and lignin of the cell walls (Marmioli et al. 2005). This strong affinity for the cell wall components may explain the low mobility of Pb in comparison with other metals such as cadmium, of which only 56 % is adsorbed into the cell constituents (Wierzbicka et al. 2007). This could explain why Pb is less toxic in plant cells than cadmium (Seregin et al. 2004).

### 3.1.2 Symplastic Pathway

The quantity of Pb passing along this route is marginal in comparison with the apoplastic pathway, and few studies have established it (Lane and Martin 1977). Yet in non-lethal doses, Pb only penetrates the symplast in areas in which cells are dividing actively, such as in the apical area (Tung and Temple 1996b) or in the protoderm (Wierzbicka 1998). Young cells do not yet possess a secondary wall and their primary wall is very thin. Access to the cell membrane is thus facilitated in these parts of the root. Studied that noted the presence of Pb in the symplast were not able to reveal the presence of Pb in its free state in the cytoplasm. Symplastic Pb may be found confined to certain cell compartments, such as vacuoles (Kopittke et al. 2008; Jiang and Liu 2010), dictyosomal vesicles (Malone et al. 1974), and vesicles of the endoplasmic reticulum (Wierzbicka et al. 2007) or plasmatabules (Wierzbicka 1998).

In lethal doses, Pb penetrates all the radicular tissues, and the cell membranes no longer appear to play their role of physical barrier (Seregin et al. 2004). In such concentrations, Pb causes the disorganisation of the membranes. It is then able to penetrate massively into the cytoplasm, the nucleus and the various organelles, including those with double membranes, such as the mitochondria (Malecka et al. 2009).

## 3.2 *Detoxification Mechanisms*

Marmioli et al. (2005) suggested that plants possess two mechanisms for the sequestration/detoxification of Pb:

- one being constitutive, corresponding to Pb binding to the cell components
- the other being inducible, corresponding to molecules capable of chelating metal (such as phytochelatins).

### 3.2.1 Constitutive Mechanisms

Pb adsorption on cell constituents appears to play a keyrole in the restriction of Pb toxicity (Krzeslowska 2011). It is worth noting that the presence of Pb enhances this phenomenon. Indeed, lead exposure increased synthesis of polysaccharides causing a significant thickening of cell walls. This thickening increases the size of the physical barrier constituted by cell walls and thus limits access to the cell membrane. It also creates new sites for potential attachments of Pb, and thus increases the capacity of extracellular sequestration.

Pb also stimulates callose deposits in cell walls (Rucinska-Sobkowiak et al. 2013) which is known to be impermeable to metal ions (Hall 2002). However, the barrier role of callose against Pb ion penetration appears to be less obvious than previously believed (Samardakiewicz et al. 2012).

### 3.2.2 Inducible Mechanisms

#### General Mechanisms

While Pb is mainly found in insoluble form, only 2.4 % of the total concentration is in a soluble form (Wierzbicka et al. 2007). So, inducible mechanisms appear to be emergency mechanisms when the metallic stress becomes too much. They are more energetically costly for the plant than the constitutive mechanisms and are therefore generally used for a limited period.

Most of Pb which enter the symplast are removed by the activity of efflux pumps present in the plasma membrane. These transporters are constitutive, but transcriptome analysis showed that their gene expression is stimulated by Pb. Thus, several ABC (ATP-binding cassette) transporters, such as AtATM3, AtPDR8, or AtPDR12 in *Arabidopsis* (Lee et al. 2005; Kim et al. 2006, 2007), have been identified as being involved in resistance to Pb.

Pb sequestration occurs very rapidly in the symplast, and thus restricts the access of Pb to some sensitive sites (Wierzbicka et al. 2007). Furthermore, Pb found into plasmatabules is swiftly excreted through the cell walls in the intercellular spaces (Wierzbicka 1998). This rapid and efficient detoxification mechanism also partially explains the fact that Pb-induced toxicity is less harmful than cadmium-induced toxicity (Wierzbicka et al. 2007).

The various intracellular sequestration mechanisms mentioned above (see 3.1.2) have not been extensively studied. P1B-2 subgroup of the P-type ATPase family, which is involved in metal transport, could play a role in Pb sequestration. In particular, the AtHMA3 protein is involved in Pb detoxification by participating in its vacuolar sequestration (Morel et al. 2009).

Pb may be transported in cell compartments after it has been bound to organic molecules. Although suspected in the case of Pb, this detoxification mechanism has not yet been clearly demonstrated, unlike that of other metals (Hall 2002). Wierzbicka et al. (2007) showed that soluble Pb was partly found in a complex with histidine, an amino acid described as participating in the detoxification of nickel (Hall 2002). This soluble fraction may also be linked to constituents possessing thiol groups such as cysteine (Vallee and Ulmer 1972) or reduced glutathione (GSH; Singh et al. 2006), which plays a central role in plant tolerance (Brunet et al. 2009; Gupta et al. 2010).

#### Phytochelatins

Pb is known to stimulate the production of phytochelatins (PCs) and to enhance PC synthase (PCS) activity (Cobbett and Goldsbrough 2002; Clemens 2006a, b). PCs seem to play an important role in the Pb tolerance of plants and participate in their detoxification (Gupta et al. 1995; Piechalak et al. 2002). PCs sequester soluble Pb present in the cytoplasm before transporting it into the vacuoles (Piechalak

et al. 2002; Seregin et al. 2002). However, the mechanism regulating the passage of the Pb-PC complex through the tonoplast is not yet known. Indeed, the recently discovered vacuolar PC transporters AtABCC1 and AtABCC2 which confer tolerance to As(III), Cd(II) and Hg(II), are not involved in Pb sequestration into the vacuoles (Park et al. 2012).

Although the principal role of the PCs seem to chelate metals (Clemens et al. 2002), their roles in metal detoxification may be more complex. In fact, they could participate not only in the translocation of cadmium from roots to shoots (Gong et al. 2003) but also from shoots to roots (Mendoza-Cozatl et al. 2008) The possibility that some specific PCs, distinct from those involved in vacuolar sequestration, could be involved in long distance transport of Pb remains debatable

### Metallothioneins

Pb can be sequestered by metallothioneins (MTs) showing homologies with yeast or human metallothioneins (Freisinger 2008). These MTs, that play a fundamental role in metal detoxification in animals, have only been studied to a small degree in plants, due to the preponderant role played by PCs (Cobbett and Goldsbrough 2002). Nevertheless, some authors have shown that Pb activates several class MT genes (Xu et al. 2007; Liu et al. 2009), while others demonstrated the Pb binding properties and detoxification ability of MTs (Xu et al. 2007; Huang et al. 2011; Fernandez et al. 2012). However, this gene activation could also be linked to the Pb-induced oxidative stress as recent works show that MTs are also involved in ROS scavenging (Hassinen et al. 2011).

### ***3.3 Translocation to the Aerial Parts of the Plant***

Certain plant species are capable of transferring large amounts of Pb to the aerial parts (Arshad et al. 2008; Gupta et al. 2013). However, slight metal translocation seems to be quite a common phenomenon in other plant species. As previously mentioned, this phenomenon is largely due to the very large amount of Pb immobilised in insoluble forms. However, it appears that the physical barrier constituted by the endodermis also plays an important role. In fact, the Pb that mostly passes along the apoplastic pathway is blocked by the Casparian strips in the endodermis. In order to go with the water flow, it must travel along the symplastic pathway through the filter constituted both by the permeability of the cell membranes and the cytoplasm sequestration/detoxification systems.

Pb absorbed by the epidermis and root hairs penetrates into cortical tissues, but does not seem to be capable of passing through the endodermis (Seregin et al. 2004). The histological localization of Pb in the roots is characteristic of a

molecule that is incapable of passing through the endodermic barrier (Seregin et al. 2004). This hypothesis is reinforced by the fact that monocotyledons possessing a more permeable endodermis, transfer greater quantities of Pb towards their aerial parts, than do the dicotyledons. Pb mostly penetrates into the central cylinder via the apex, a region in which the endodermis has not yet formed (Tung and Temple 1996b; Seregin et al. 2004). Consequently, it would appear that in non-lethal doses, Pb translocation to the aerial parts originates solely from the radicular apex.

Several authors have shown that the water flow bypassing the endodermis barrier constitutes only a small part of the total flow (less than 1 %) migrating to the aerial parts (Skinner and Radin 1994). These results seem to agree with the ratio, observed during exposure to low or average concentrations of Pb, between the Pb contained in the shoots and the roots that is less than 1 % (Huang and Cunningham 1996). This theory is supported by the fact that the water flow increases significantly in damaged roots (Moon et al. 1986), a phenomenon comparable to that observed by Seregin et al. (2004) for Pb in damaged roots. Thus, during exposure to high concentrations, the proportion of Pb concentrations in the aerial parts and those contained in the roots exceeded 20 % (Singh et al. 1997).

Once Pb has penetrated into the central cylinder, it can once again resume its route through the apoplastic pathway (Tung and Temple 1996a, b). Pb then uses the vascular system by following the water flow to the leaves, in which water evaporates and where it accumulates (Tung and Temple 1996a). When it passes into the xylemic sap, Pb may be compounded with amino acids such as histidine and with organic acids such as citric, fumaric and malic acids (Shahid et al. 2012b; Ghnaya et al. 2013) or with the PCs, as discussed previously. It may also be transferred, mostly in an inorganic form, as is the case for cadmium (Ueno et al. 2008).

## 4 Lead Effects on Plants

Actually, the small proportion of Pb penetrating into the symplast produces a great variety of harmful effects on plants (Singh et al. 1997; Seregin and Ivanov 2001; Pourrut et al. 2011b). However, mechanisms of Pb toxicity remain unclear. The set of observed macroscopic effects could result from:

- the interaction of Pb with various cell components and macromolecules (proteins, DNA, etc.);
- disruption to numerous physiological processes such as regulation of the water status, mineral nutrition, respiration or photosynthesis.



## **4.1 Effects on the Cell Constituents**

### **4.1.1 Effects on the Cell Envelopes**

As previously stated, Pb may bind itself on the constituents of the cell walls or membranes. This fixing leads to a cell wall mineralisation (Wierzbicka 1998) which is better known in the case of calcification and silicification. Cell wall mineralisation produces a change in the cell wall physical and chemical properties. Especially, it affects cell wall plasticity which modulates numerous cell mechanisms such as division or elongation.

Pb not only disrupts the external envelopes by binding to them, but also disorganises the network of microtubules (Wierzbicka 1998; Eun et al. 2000) which play a major role in numerous processes such as intracellular traffic, mitosis and morphogenesis, determining the polarity of growth and the shape of the cells. Consequently, the emergence of abnormal cell shapes and uneven thickness of the cell wall is observed (Wierzbicka 1998). It is also interesting to note that this effect is characteristic of Pb, and has not been observed in the case of other metals such as copper or aluminium which also inhibit cell growth (Eun et al. 2000).

Although the action mechanisms are not yet known, Pb produces serious changes in the lipid compositions of the various cell membranes (Stefanov et al. 1995a; Singh et al. 2010). Furthermore, Pb induces lipid peroxidation via the generation of ROS (Singh et al. 2010; Gupta et al. 2011; Shahid et al. 2012a). These changes to the membrane lipids lead to the appearance of abnormal cell structures, especially alterations in the cell membrane (Islam et al. 2007), as well as in the organelles such as the mitochondria and peroxisomes (Małeczka et al. 2008) or in the chloroplasts (Weryszko-Chmielewska and Chwil 2005; Islam et al. 2007). Strong concentrations cause membrane ruptures, leading to cell death in the long term.

### **4.1.2 Effects on Proteins**

#### Effects on the Protein Pool

The action of Pb on the total protein content is unclear, although high concentrations may reduce the protein pool (Jana and Choudhuri 1982; Mishra et al. 2006; Singh et al. 2010). This pool reduction appears to result from various actions of Pb:

- modification of the gene expression (Kovalchuk et al. 2005) and an increase in ribonuclease activity (Jana and Choudhuri 1982; Gopal and Rizvi 2008);
- oxidation of proteins (Wang et al. 2008);
- stimulation of protease activity (Jana and Choudhuri 1982);
- reduction in the free amino acid content (Xiong et al. 2006), correlated to a serious disruption in the nitrogen metabolism. An increase is noted, however, in

certain amino acids such as proline which appears to play an important role in the plant tolerance to Pb (Saradhi and Saradhi 1991).

Lower Pb concentrations could, on the contrary, increase the total protein content (Mishra et al. 2006). This protein accumulation, which is limited to the early days of exposure, may be due to a synthesis of defence proteins against the metal stress, in particular with proteins playing a role in the maintenance of the redox status (ascorbate, GSH) or in the metal sequestration (GSH, PC).

Pb also changes the proteome profile (Bah et al. 2010; Walliwagedara et al. 2010), which may be correlated with a change in the transcriptome profile (Kovalchuk et al. 2005; Liu et al. 2009).

### Inactivation Mechanisms

Pb is able to inhibit enzyme activity and possesses an inactivation constant ( $K_i$ ) of between  $10^{-5}$  and  $2.10^{-4}$  M. (Seregin and Ivanov 2001). This inhibition is generally the consequence of interaction between Pb and enzyme thiol groups (Seregin and Ivanov 2001). This is the case for more than 100 enzymes, especially rubisco and nitrate reductase. This enzymatic inactivation is due either to metal-binding in the catalytic domain, or elsewhere on the protein, producing a change in its tertiary structure. Pb can also bind to the protein carboxyl group and produce the same effects.

The second interaction mechanism with proteins concerns the metalloenzymes. Pb can disrupt the plant absorption of minerals that are essential for these enzymes, such as zinc, iron and manganese (see 4.3). Like the other divalent cations, it can also substitute itself for these metals and thus cause the inactivation of enzymes. This is particularly true of  $\delta$ -aminolevulinic acid deshydratase (ALAD), which participates in the biosynthesis of chlorophyll.

The last main interaction mechanism with proteins is an indirect one, resulting from the oxidative damage of ROS on proteins. However, it is difficult to evaluate the oxidative stress role in the Pb-induced inactivation of enzymes since no study has been done on this subject.

### Activation Mechanisms

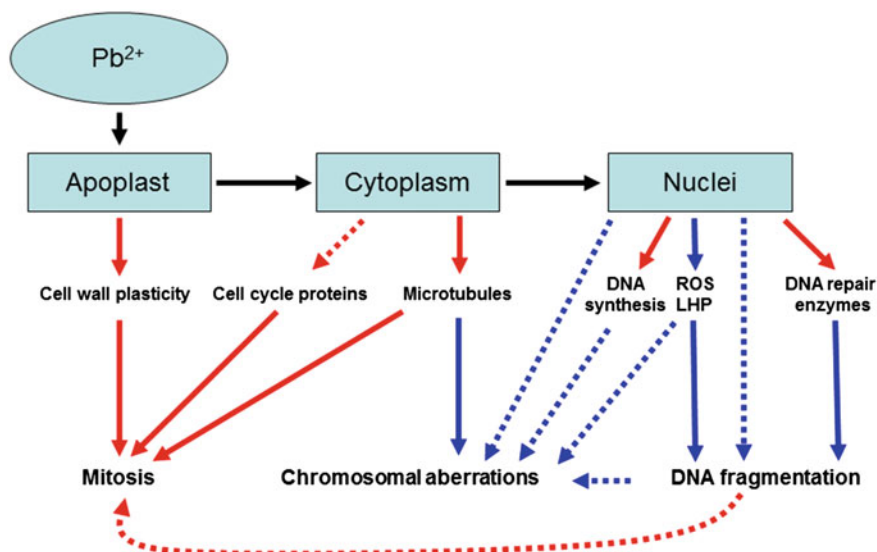
Pb stimulates the activity of certain enzymes, but the mechanisms of enzyme activation are not known. Pb may modulate the gene expression of these enzymes or restrict the activity of their inhibitors (Seregin and Ivanov 2001). However, in the case of proteases and nucleases, their activation may be the consequence of an increase in the concentration of their substrates (due to the action of Pb) rather than a direct interaction with Pb.

### 4.1.3 Antimitotic and Genotoxic Action of Lead

The antimitotic effect of Pb is one of the best-known aspects of Pb toxicity (for review, see Patra et al. 2004). The first interaction mechanism is the mineralisation of the membranes and cell walls, which reduces cell division (Fig. 1). The second interaction mechanism is the disruption of the microtubules whose organisation is essential for mitosis. Pb exposure essentially induces disturbances in the G2 and M stages of cell division, leading to the formation of abnormal cells at the colchicin-mitosis stage. This phenomenon is accentuated by the direct or indirect interactions of Pb with the proteins involved in the cell cycle, such as the cyclins, whose activity is indirectly dependent on the GSH concentration. The spindle activity disturbances caused by Pb may be transient, and in some cases the mitotic index returns to initial level. Furthermore, low doses of Pb do not seem to have a significant effect on mitosis (Pourrut et al. 2011a; Shahid et al. 2011).

Even at low concentrations, Pb induces chromosomal aberrations (Shahid et al. 2011, 2012a): formation of chromosome bridges during the anaphase, loss of excentric fragments during homologous recombination, chromosome fragmentation and formation of micronuclei. Pb is also known to induce simple and double strand breaks (Gichner et al. 2008; Pourrut et al. 2011a) and microsatellite instability (Rodriguez et al. 2013).

However, with the exception of Pb action on tubulin (Fig. 1), little is known about Pb-induced genotoxic mechanisms on plants. Pb may penetrate into the



**Fig. 1** Representation of proven (solid arrows) and potential links (dotted arrows) between lead, mitosis and genotoxicity, Red arrow negative interaction, blue arrow positive interaction, black arrow movement of Pb. LHP lipid hydroperoxides

nucleus (Małacka et al. 2008) and bind directly to the DNA or indirectly to proteins fixed on the DNA. However, it would appear that Pb has no direct genotoxic action on DNA (Valverde et al. 2001; Pourrut et al. 2011a). Many of the Pb genotoxic effects in mammalian cells are mediated by ROS and/or lipid-soluble by-products of oxidative stress such as MDA (Valverde et al. 2001; Xu et al. 2008). This hypothesis is supported by the results obtained by Pourrut et al. (2011a) on *Vicia faba* roots. Moreover, Pb binding on DNA disrupts DNA repair and replication. Pb affects these mechanisms by replacing zinc in the zinc finger pattern of numerous DNA repair and replication enzymes (Gastaldo et al. 2007).

## 4.2 Water Status

Numerous studies have shown major disruptions to the water status of Pb-exposed plants: reduction in transpiration and water content (Barcelo and Poschenrieder 1990; Brunet et al. 2009). The reduction in leaf growth, leading to a reduction in the foliar area, could explain these phenomena. Moreover, the stomata of Pb-exposed plants are smaller (Weryszko-Chmielewska and Chwil 2005). Nevertheless, the plants seem to be able to adapt to these two effects by developing a greater density of stomata (Kosobrukhov et al. 2004; Weryszko-Chmielewska and Chwil 2005).

As previously mentioned, Pb can reduce cell wall plasticity, and consequently affect the water potential of the cell. This phenomenon is accentuated by the reduction observed in the concentrations of molecules that maintain cell turgescence such as sugars and amino acids (Barcelo and Poschenrieder 1990). This change in the cell water potential, especially in the guard cells, poses problem in regulating the opening and closing of the stomata. To maintain cell turgescence, the plant massively synthesises osmolytes, and especially proline (Saradhi and Saradhi 1991; Zhou et al. 2010).

The presence of Pb results in a high accumulation of abscissic acid (ABA) in the roots and aerial parts (Parys et al. 1998; Atici et al. 2005), causing the stomata to close (Kosobrukhov et al. 2004; Weryszko-Chmielewska and Chwil 2005). This closing seriously restricts the exchange of gases with the atmosphere, and consequently the loss of water through transpiration (Parys et al. 1998). The plant transpiration via pores is also restricted by the deposit of a cuticle layer on the leaf surface (Weryszko-Chmielewska and Chwil 2005). On one hand, this restriction on transpiration limits Pb translocation from roots to shoots (Wang et al. 2013). On the other hand, this phenomenon restricts water flow through the plants and consequently the circulation of the xylem sap.

### 4.3 Mineral Nutrition

The main consequence of the water flow limitation is the restriction the soil solution absorption by the roots. Thus, it induces *de facto*, a change in the mineral elements absorption. Although data are insufficient to allow a definitive conclusion to be drawn, Pb affects nutrient uptake (Pourrut et al. 2011b). It emerges clearly that Pb reduces nutrient concentration in shoots (Krupa et al. 2002; Cannata et al. 2013). This is particularly true of divalent ions such as  $Zn^{2+}$ ,  $Mn^{2+}$ ,  $Mg^{2+}$ ,  $Ca^{2+}$  (Pourrut et al. 2011b). However, it is not possible to determine whether this reduction is due to a reduced root uptake, a reduced translocation to the aerial parts or a mobilisation of these elements through the plant. While the response of the aerial parts seems to be shared, the distribution of these elements in the roots seems to depend on the type of plant or the intensity of the stress.

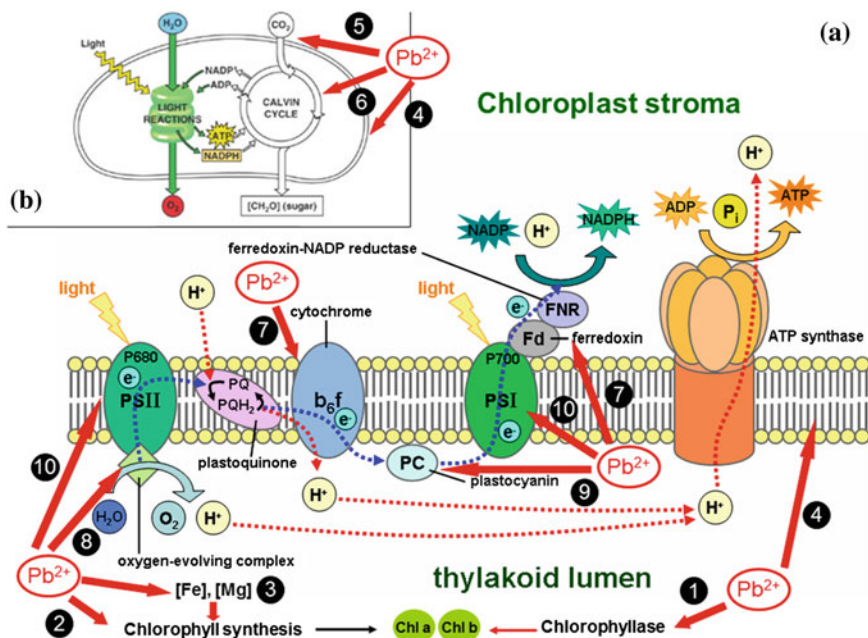
Potassium is the only ion the concentration of which reduces in the roots, without this having a formal effect on its concentration in the aerial parts. The interaction with Pb seems to be really specific and could be explained by their similar radii (1.29 Å for  $Pb^{2+}$  against 1.33 Å for  $K^+$ ). These ions may compete for entry into roots through potassium channels.

Pb induces a general reduction in inorganic nitrogen content in the whole plant. This could be explained by (i) the disruption of its root uptake, (ii) the reduced activity of nitrate reductase or (iii) the increase of the synthesis of amino acids (proline) and peptides (GSH) playing a role in Pb tolerance and detoxification.

### 4.4 Photosynthesis

In plants, Pb strongly inhibits the photosynthesis process. In particular, the photosynthetic yield is very sensitive and may reduce by more than 50 % (Bazzaz et al. 1975; Poskuta et al. 1988). This inhibition can be explained by the reduction in the chlorophyll and carotenoid content that has generally been observed (Kosobrukhov et al. 2004; Gopal and Rizvi 2008). Chlorophyll b seems to be more sensitive than chlorophyll a (Stiborova et al. 1986; Vodnik et al. 1999).

The reduction mechanisms in pigment content have been partially explained. Pb induces chlorophyllase activity in the leaves (Drazkiewicz 1994; Fig. 2.1). but the main mechanism seems to be inactivation of the synthesis of these pigments (Gupta et al. 2009; Ceneci et al. 2010). Pb interacts (i) directly by substituting itself for the divalent ions bound to metalloenzymes such as ALAD, which is a key metalloenzyme for chlorophyll biosynthesis and in which the  $Zn^{2+}$  ion is replaced by  $Pb^{2+}$  (Fig. 2); (ii) indirectly, by inducing a lack of ions (Fig. 2). It is accepted that chlorosis is generally a sign of a lack of iron that is indispensable for synthesising chlorophyll (Foy et al. 1978). Recently, Shahid et al. (2012a) demonstrated that Pb-induced reduction in pigment contents of *Vicia faba* leaves during 24 h of exposure might be due to the involvement of fast mechanisms, such as ROS production.



**Fig. 2** Schematic and simplified representation of effects of Pb on photosynthetic chain (a) and chloroplast (b). Pb (*I*) induces chlorophyllase activity; disrupts (2) directly chlorophyll synthesis or (3) indirectly by limiting nutrient uptake, and (4) electron transport chain. Pb limits  $CO_2$  assimilation by (5) closing stomata and (6) inhibiting enzymes involved in the Calvin Cycle. By causing a nutrient shortage, Pb heavily disrupts the metalloproteins or enzymatic complexes of the photosystems: (7) cytochromes  $b_6f$  and ferredoxines, (8) oxygen-evolving complexes, (9) plastocyanines and (10) chlorophylls

The reduction observed in photosynthetic activity is often far greater than the reduction observed in pigment content. Thus, Pb may act at other levels (Bazzaz et al. 1975; Stiborova et al. 1986). At the structural level, as previously mentioned, Pb produces deformities in the structures of the chloroplasts, since these are more sensitive than the mitochondria (Weryszko-Chmielewska and Chwil 2005; Islam et al. 2007). It also produces a change in lipid composition in the chloroplast membrane (Stefanov et al. 1995b), whose physico-chemical properties are changed as a result. Furthermore, when Pb penetrates into a chloroplast, it can bind with the chloroplast membrane. These various types of interactions disrupt, in particular, the flow of electrons in the electron transport chain (Fig. 2). Qufei and Fashui, (2009) showed that  $Pb^{2+}$  accumulated in PS II and damaged its secondary structure, inhibiting energy transfer among amino acids within the PS II protein-pigment complex, and reduced energy transport from tyrosine residue to chlorophyll a.

By causing closure of the stomata, Pb considerably restricts the flow of gases between the leaves and the atmosphere (Fig. 2). This reduction in the flow of incoming CO<sub>2</sub> is considered to be the main reason for the significant reduction observed in the fixation of CO<sub>2</sub> (Bazzaz et al. 1975; Parys et al. 1998; Romanowska et al. 2002). Using the same interaction mechanisms as previously described, Pb can also inhibit certain enzymes involved in the Calvin Cycle (Fig. 2.6; Vallee and Ulmer 1972).

Due to the reduction in the concentration of certain minerals observed in leaves, Pb heavily disrupts the photosystems. These contain Fe-S centres that are also present in several molecules involved in photosynthesis (cytochrome b6f, ferredoxine; Fig. 2). Furthermore, the oxygen-evolving complex contains four manganese atoms (Fig. 2), plastocyanines contain copper (Fig. 2) and chlorophylls contain tetrapyrrole/magnesium (Fig. 2). Pb may also interact directly with these molecules, by substituting itself for their divalent ions.

## 4.5 Respiration

Unlike the photosynthetic activity, the effect of Pb on respiratory activity has not been much studied, and solely on leaves (Seregin and Ivanov 2001). The leaves of Pb-exposed pea plants saw their CO<sub>2</sub> concentrations increase significantly (Parys et al. 1998). This increase is the result of a reduction in photosynthetic activity and an increase in respiration. Leaf respiration is the result of dark (mitochondrial) respiration and of the photorespiration performed by the Rubisco in the chloroplasts. Romanowska et al. (2002) showed that the increase in respiration, induced by Pb, was due solely to dark respiration. Photorespiration was not affected (Romanowska et al. 2002) or even inhibited (Poskuta et al. 1988). The overall increase in respiration leads to an increase in the concentration of ATP, which could correspond to a high need for energy in the plant to cope with Pb.

Poskuta et al., (1996) suggested that, in conditions where there is a lesser fixation of CO<sub>2</sub>, the stimulation of dark respiration could be explained by a transfer of the redox equivalents produced by photosynthesis to the mitochondria, thus increasing respiration. Several studies have shown that this is a common phenomenon, even under normal cultivation conditions (see for review Kromer 1995).

In high concentrations, Pb has a strongly inhibiting effect on the respiratory chain. This inhibition may be the consequence of the fixation of Pb on the mitochondria membrane, disrupting the transport of electrons (Bittell et al. 1974) and could lead to the uncoupling of the phosphorylation step, as is the case with cadmium and zinc (Romanowska 2002).

## 5 Lead and Oxidative Stress

### 5.1 Lead-Induced Oxidative Stress

In aerobic organisms, the production of a large amount of ROS and the resulting generation of oxidative stress are among the best known and earliest aspects of Pb toxicity (Clemens 2006b; Pourrut et al. 2011b). However, the link between Pb-induced ROS and the Pb toxicity has been less studied.

Direct measurements of ROS show that Pb exposure increases the production of H<sub>2</sub>O<sub>2</sub> and of superoxide radical O<sub>2</sub><sup>-</sup> (Pirslova et al. 2011; Kaur et al. 2012; Wang et al. 2012). An indirect estimation of the ROS production is also possible by measuring the activity of the antioxidant enzymes. As a general rule, the superoxide dismutases, the peroxidases (ascorbate peroxidases, guaiacol peroxidases) and glutathione reductases are activated, regardless of the Pb stress and the length of time it lasts (Pourrut et al. 2011b). The case of catalase is more complex, since half the publications report an inhibition and the other half show an increase in the activity through Pb (Pourrut et al. 2011b). The inhibition of catalase activity was found in various levels of stress, both in plants and in yeasts and humans. Since catalases are metalloenzymes, this inhibition could be due to direct interaction with Pb.

The idea of a Pb-induced oxidative burst is reinforced by the study of lipid peroxidation, which is shown to be correlated to the increase in antioxidant activities (Singh et al. 2010; Gupta et al. 2011; Shahid et al. 2012a). Pb also stimulates the gene expression of lipoxygenases (Pourrut et al. unpublished) as well as their activity (Huang and Huang 2008; Huang et al. 2012).

### 5.2 Role of NADPH-Oxidase in Lead-Induced Toxicity

Under normal conditions, the main sources of ROS in plants are the electron transport chain of chloroplasts and mitochondria (Foyer and Noctor 2003). However, under metal stress, alternative enzymatic sources could play a key role in ROS generation.

#### 5.2.1 Activation of NADPH-Oxidase

Since the past decade, researchers have begun to show an interest in the sources of ROS during metal stress. The backup generation systems for ROS do not make it easy to determine the source responsible for ROS production under Pb stress. The primary oxidative burst induced by metals may be apoplastic and of enzymatic origin (Mika et al. 2004). Several studies highlighted the role of membrane NADPH-oxidases (NOX) in the response to cadmium, nickel and copper

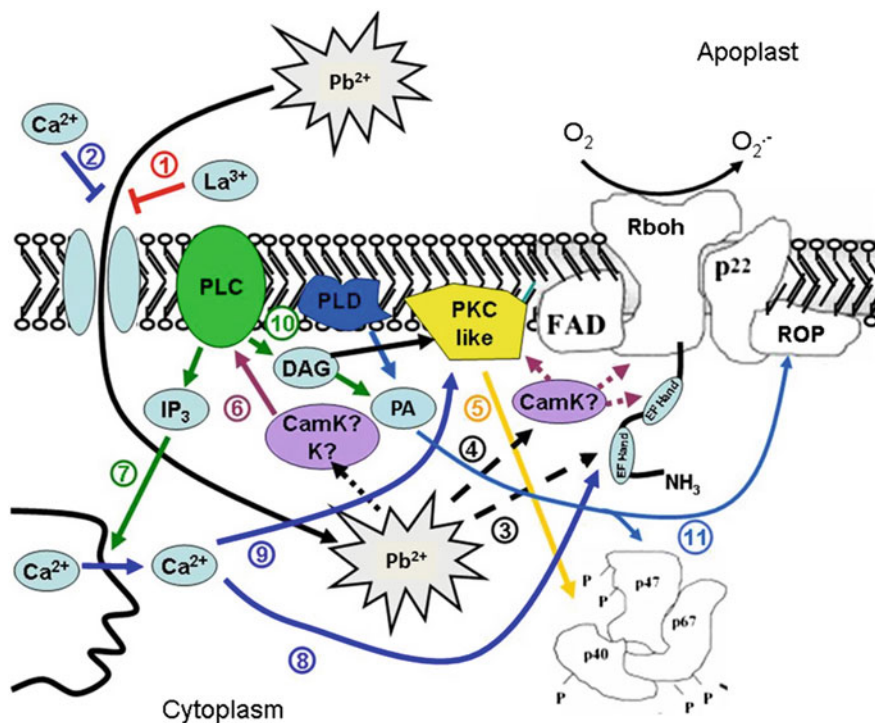


(Quartacci et al. 2001; Olmos et al. 2003; Romero-Puertas et al. 2004; Hao et al. 2006). Pourrut et al. 2008 were the first to clearly show the involvement of NOX in the primary oxidative burst caused by Pb, as previously shown for other metals. These results were confirmed later by the works of Huang and Huang (2008), Pourrut et al. (2011b) and Wang et al. (2012).

Plant NADPH-oxidases (also called Rboh for respiratory burst oxidase homologs) are homologs to the NADPH enzymatic complex of animal cells (see for review Glyan'ko and Ischenko 2010). In plants, the activation of NOX seems to be a universal response to metal stress. The mechanisms of this activation have not been totally elucidated, and the role of calcium is controversial. Pourrut et al. (2008) showed that lanthanum, an inhibitor of voltage-dependent calcium channels and antiports  $\text{Ca}^{2+}/\text{H}^{+}$  (Bush 1995), completely eliminated the oxidative burst. This result, also observed in the case of cadmium (Olmos et al. 2003; Romero-Puertas et al. 2004; Garnier et al. 2006), led Romero-Puertas et al. (2004) to conclude that an extracellular calcium input caused NOX activity. However, calcium saturation of the external medium, as well as the use of  $\text{La}^{3+}$  inhibits  $\text{O}_2^-$  production induced by cadmium (Olmos et al. 2003; Garnier et al. 2006) as well as by Pb (Pourrut et al. 2008) and nickel (Hao et al. 2006). Moreover, the use of A23187, a membrane calcium ionophore, produced no significant effect (Pourrut 2008). These two data contradict the Romero-Puertas hypothesis and suggest that the penetration of these metals occurs non-specifically and via pathways that are sensitive to lanthanum (Fig. 3).

The EF hand motifs of the Rboh protein enable direct activation of the protein by calcium (Sagi and Fluhr 2001). Pb has a strong affinity with these motifs and can come into competition with calcium to bind with it (Habermann et al. 1983). Once Pb has penetrated the symplast, it has the potential to interact with Rboh EF hand motifs (Fig. 3), and thus directly activate the NOX. Pourrut et al. (2008) have nevertheless highlighted the involvement of kinase proteins (PK) in Pb-induced NOX activation and revealed a calmodulin-dependent step (Fig. 3). Unlike animal cells, plant cells possess few  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (CamK; Harmon et al. 2000). The lack of data concerning these enzymes in plants does not allow discussing this calmodulin-dependent stage. Yet the total inhibition of superoxide anion production by staurosporine, a wide-spectrum PK inhibitor, cannot be imputed to inhibition of a hypothetical CamK (Pourrut et al. 2008). Two types of PK play an important role in NOX activation in human cells. The first type is protein kinase C (PKC) that phosphorylates cytosolic components, causing the formation of the enzymatic NOX complex (Fig. 3). There is no PKC in plants, despite the fact that PKC activity has been shown (Talwar et al. 2012), suggesting the presence of a PKC-like kinase. In *Vicia faba* roots untreated with Pb, exposure to a diacylglycerol analogue the 12-O-tetradecanoylphorbol-13-acetate (TPA), a well-known PKC activator in animal cells (Pinelli et al. 1994), induces an oxidative burst, indicating the presence of PKC-like kinase and its involvement in the regulation of the NOX complex (Pourrut 2008).

The other PK type corresponds to the PK involved in phosphorylation and the activation of phospholipase C (PLC; Fig. 3). Garnier et al. (2006) showed that this



**Fig. 3** Schematic representation of the various potential routes for the regulation of NOX in plants following exposure to Pb. Lead entry into cell could be lanthanum-sensitive (1) and non-specific (2). Once Pb has penetrated the symplast, it could activate Rboh (3) directly by binding EF hand motifs or (4) indirectly via a  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (CamK)-dependent step. Then, a protein kinase C (PKC)-like enzyme phosphorylates cytosolic components, causing the formation of the enzymatic NOX complex (5). A kinase-dependent step (6) leads to the activation of phospholipase C (PLC) which could play a central role in NOX activation. PLC activity generates the formation of inositol triphosphate ( $\text{IP}_3$ ) and diacylglycerol (DAG).  $\text{IP}_3$  is responsible for the release of  $\text{Ca}^{2+}$  contained in cell reservoirs (7) which could directly activate NOX through bounding with EF hand motifs (8) or indirectly through activating PKC-like kinase (9). The DAG formed could also activate PKC-like directly (10). Phosphatidic acid (PA) formed through transformation of the DAG could also activate the NOX enzymatic complex (11)

enzyme plays a central role in NOX activation under cadmium stress. Its activity generates the formation of inositol triphosphate ( $\text{IP}_3$ ) and diacylglycerol (DAG).  $\text{IP}_3$  is responsible for the release of  $\text{Ca}^{2+}$  contained in cell reservoirs (Fig. 3.7; Dong et al. 2012). The sudden increase in the intracellular concentration of calcium could directly activate NOX through bounding with EF hand motifs (Fig. 3) or indirectly through activating PKC-like kinase (Fig. 3). The DAG formed could also activate PKC-like (Fig. 3).

Another source of NOX activation could be the formation of phosphatidic acid (PA). The way in which it acts has not yet been clearly defined, but it appears to participate in the phosphorylation of the protein p47<sup>phox</sup> causing its migration to the membrane and its combination with membrane proteins of the NOX enzymatic complex (Waite et al. 1997; Fig. 3.11). Park et al. (2004) have shown that AP activates GTPase ROP protein, thus regulating NOX activity (Fig. 3). The AP could be formed through transformation of the DAG or activation of a different phospholipase, PLD. This enzyme is involved in NOX activation in tobacco plant cells exposed to copper (Yu et al. 2008).

### 5.2.2 Implication of NADPH-Oxidase in Lead-Induced Toxicity

Previous works on plant NOX were often confined solely to identifying the initial source of the oxidative burst and do not really make it possible to understand the role played by this enzyme in metal toxicity. Pourrut et al. (2008), (2011a) clearly showed that NOX plays a central role in Pb toxicity. At a macroscopic level, the use of DPI (a well-known NOX pharmacological inhibitor) removed the browning phenomenon observed on *Vicia faba* roots and reduced apical necrosis (Pourrut 2008). DPI also totally inhibited Pb genotoxicity and the formation of micronuclei (Pourrut et al. 2011a).

### 5.2.3 Alternative Sources of ROS

If NOX seems to play a key role in the first step of oxidative burst, the simultaneous production of ROS by several enzymatic sources should also not be ruled out (Mika et al. 2004). Once the first oxidative burst has passed, a second often occurs which may be generated after several hours of exposure to stress, either by the same production systems, or by other systems independent of the first (Mika et al. 2004). In fact, extracellular peroxidases may play a role in the oxidative burst induced by cadmium, copper, cobalt and aluminium (Simonovicova et al. 2004a), and the oxalate-oxidases in the response to cadmium and aluminium (Simonovicova et al. 2004b; Simonovicova et al. 2005). Groppa et al. (2007) also showed that the activity of amine-oxidases in relation to copper was modulated by cadmium and copper. However, these authors did not show whether these enzymes participated in the oxidative outbreak.

Oxidative stress may continue over time and space, with the release of lipid peroxides and the production of intracellular ROS, as for example in the mitochondria (Garnier et al. 2006).

## 6 Conclusion and Perspectives

The behaviour of Pb in soil depends on a large number of biogeochemical parameters. Despite the exact mechanisms of entry being still unclear, Pb mainly penetrates into plants through the roots. Due to its strong affinity for cell wall and membrane constituents and to plant detoxification systems, most of Pb is generally sequestered into roots. Excessive Pb accumulation in plant tissue impairs various morphological, physiological and biochemical functions in plants, either directly or indirectly, and induces a range of deleterious effects. Oxidative stress which is one the most well-known effects of Pb exposure seems to play a central role in Pb toxicity. Moreover, at least in the early steps, Pb-induced oxidative stress is strongly associated to NADPH oxidase activation. Despite numerous studies, the lack of data leads to poor understanding of the molecular mechanisms of these phenomena. Further researches are needed to:

- fully elucidate pathways of Pb entry and fluxes in plants,
- clearly understand molecular aspects of Pb toxicity,
- figure out mechanisms of plant defence against Pb,
- look further into the implication of ROS in Pb toxicity and
- precise the role of NADPH oxidase in Pb-induced oxidative stress.

A better understanding of molecular mechanisms involved is not only essential to figure out Pb uptake, toxicity and detoxification in plants, but also to improve phytomanagement of Pb contaminated sites.

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