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Lead stress triggers the synthesis of protecting salicylic acid through polyamine accumulation in the Pb-resistant species *Piptatherum miliaceum* (L.) Cosson

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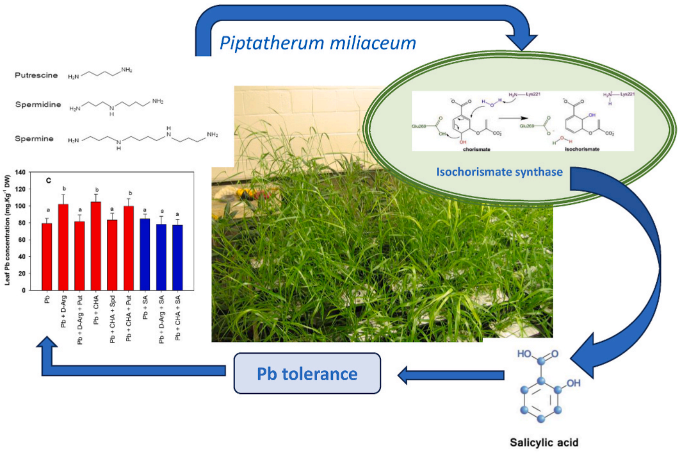
HIGHLIGHTS

- *Piptatherum miliaceum* can cope with high external doses of Pb in nutrient solution.
- Polyamines and salicylic acid contribute to lead tolerance in *Piptatherum miliaceum*.
- Ethylene is involved in lead toxicity in relation to lead absorption and accumulation.
- Spermine contributes to salicylic acid synthesis in the shoots of Pb-treated plants.
- Spermine increases chloroplastic isochorismate synthase activity in leaf tissues.

GRAPHICAL ABSTRACT



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ABSTRACT

To assess the impact of polyamines and salicylic acid on the response of *Piptatherum miliaceum* to lead toxicity, plants were cultivated in nutrient solution containing 500 μM $Pb(NO_3)_2$ in the presence or absence of polyamine synthesis inhibitors (D-Arginine 5 mM, cyclohexylammonium 5 mM, methylglyoxal-bis-guanyl-hydrazone 1 mM), ethylene synthesis inhibitor (2-aminoethoxyvinyl-glycine 2 μM) and/or exogenous supplementation of 100 μM putrescine, spermidine, spermine or 10 μM salicylic acid. Although all plants remained alive until the end of the treatment, Pb toxicity induced a decrease in growth and cell viability and an increase in electrolyte leakage. Plants accumulated up to 84.2 mg kg^{-1} DW Pb in the shoots and 901.6 mg kg^{-1} DW in the roots. Lead increased endogenous concentration of polyamines and salicylic acid. It increased phenylalanine ammonia lyase activity

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Salicylic acid
Smilo grass

(PAL) in roots and shoots and isochorismate synthase activity (ICS) in shoots only. Glutathione increased in response to Pb, as well as root ascorbate and phytochelatins. All inhibitors of polyamines aggravated the deleterious impact of Pb on recorded parameters while simultaneous application of polyamines allowed to compensate this effect. Spermidine restricted root-to-shoot Pb translocation while ethylene increased Pb absorption and accumulation. Spermine stimulated ICS activity in the shoots but not in the roots, leading to high salicylic acid (SA) concentrations in photosynthetic tissues. Salicylic acid stimulated glutathione and phytochelatin synthesis in the roots. Hence, polyamines and SA play a role in Pb tolerance of *P. miliaceum* but act on distinct targets. These compounds exhibit specific interaction in Pb-stressed organs which differ between roots and shoots.

1. Introduction

For several decades, numerous human activities have been releasing large quantities of heavy metals into the environment, posing a risk to human health and stability of ecosystems. Among the elements concerned, lead is probably the most widespread and one of the most problematic. Many industrial and mining activities are responsible for lead contamination over large areas (Kumar and Prasad, 2018; Kanwar et al., 2020; Haghighizadeh et al., 2024). Human can be exposed to contaminants through breathing of suspended particles or ingestion of polluted material through the food chain. Lead acts as a physiological and neuronal toxin and is naturally carcinogenic in the human body (Meng et al., 2020; Vagnoni et al., 2024). Mining processes associated with heavy metals extensively used in industrial applications have been identified as primary contributors to soil contamination (Haghighizadeh et al., 2024). Abandoned mine areas contain different types of residues from ore processing (Robles-Arenas et al., 2006). Acid mine drainage caused by oxidation and hydrolysis of galena (PbS) induces a pH decrease allowing high Pb mobility in numerous mining zones (Margui et al., 2004). Physical and chemical approaches (electrokinetic remediation, soil washing, vapor sparging, stabilization/solidification) are available to address soil contamination. Although highly effective for heavy metals removal, they do not constitute an economical option for cleaning large surfaces of contaminated soil and may even cause disturbances in biological and physico-chemical soil properties (Han et al., 2023; Sun et al., 2023). The use of plants and associated microorganisms to remove or stabilize pollution in contaminated sites thus appears as a suitable and cheap alternative for eco-restoration of polluted areas (Kanwar et al., 2020; Alsafran et al., 2022; Wang and Delavar, 2024).

Grasses from the Poaceae family may be considered as suitable phytoremediators of heavy metals in relation to their extensive root system, capability to endure harsh environment, rapid coverage of contaminated surface and lowering of soil erosion (Patra et al., 2021). As far as lead toxicity is concerned, the pioneer tailing colonizer grass *Piptatherum miliaceum* (smilo grass) is a promising species. This plant species is adapted to acid conditions (Zavas et al., 1996) and can accumulate and tolerate Pb in its different organs (García et al., 2004). The spontaneous presence of *P. miliaceum* on acid mine tailings stimulated microbial biomass and enzyme activities contributing to soil improvement (Moreno-Barriga et al., 2017; Conesa et al., 2023). Ecological advantages of *P. miliaceum* as a pioneer species as well as intra- or inter-species interaction are well documented (Martínez-Oró et al., 2017; Parraga-Aguado et al., 2015) but the physiological basis of lead tolerance in this species remain unknown. Ierna and Mauromicale (2020) demonstrated that distinct ecotypes of smilo grass may exhibit contrasting behavior in terms of growth in harsh conditions. A better understanding of physiological processes sustaining lead tolerance may thus provide useful information allowing identification of the most promising material for phytomanagement purposes.

Lead has a detrimental impact on almost all aspects of plant metabolism and physiological processes. It reduces plant growth through a direct interference with spindle fiber polymerization during mitosis and modification of cell wall structure leading to a decrease in extensibility and cell elongation (Kumar and Prasad, 2018; Sofy et al., 2020; Abedi et al., 2022). It induces oxidative stress through the synthesis of highly reactive oxygen species (superoxide anions, hydroxyl radicals, organic hydroperoxide, singlet oxygen and peroxyl radicals) able to react with lipids and proteins and to induce DNA damages. Lead disrupts several key metabolic processes and compromises photosynthesis, biological membrane stability and selective permeability and energy metabolism (Fischer et al., 2014; Legocka et al., 2015; Kumar and Prasad, 2018; Ferrer et al., 2018; Aslam et al., 2021; Kumbhakar et al., 2023). Because lead induces a wide range of damages and act on a high number of targets, it may be hypothesized that polyvalent compounds assuming numerous regulatory and protective functions may be involved in Pb tolerance.

Polyamines (PAs) are ubiquitous small aliphatic compounds present in all eukaryotic cells. In plants, the diamine putrescine (Put) is synthesized from arginine or ornithine through the action of arginine decarboxylase (ADC; EC 4.1.1.19) and ornithine decarboxylase (ODC; EC 4.1.1.17), respectively, as illustrated in Fig. 1A. Putrescine is then converted by spermidine synthase (SpdS; EC 2.5.1.16) to the triamine spermidine (Spd) by addition of decarboxylated S-adenosylmethionine (dSAM). A subsequent addition of dSAM to Spd provides the tetramine spermine (Spm). Decarboxylated *S*-adenosylmethionine is produced from *S*-adenosylmethionine (SAM) by S-adenosylmethionine decardoxylase (SAMDC; EC 4.1.1.50). Since SAM is also a precursor of the stress-induced phytohormone ethylene, it is often considered that the pathways of polyamine and ethylene synthesis are regulated in an opposite way in response to environmental constraints (Takács et al., 2021; Jangra et al., 2023). Polyamines assume plethora of functions in plants. They are involved in plant growth and development, regulation of flowering processes, management of cell wall properties, control of cell division, regulation of photosynthesis and stomatal closure, mineral nutrition, …(Tyagi et al., 2023; Roy et al., 2024). Considering their polycationic nature at physiological pH, polyamines may also interact with negatively charged molecular targets such as membrane lipids, nucleic acids and enzymatic proteins to stabilize their structure (Jangra et al., 2023; Roy et al., 2024). Polyamines are involved in plant responses to biotic and abiotic stresses, including heavy metals (Taie et al., 2019; Spormann et al., 2021; Malik et al., 2022).

Salicylic acid (SA) has also numerous regulatory functions as plant hormone. It is a well-established molecule for systemic acquired resistance but also acts as a regulator of flowering time, growth inhibitor through attenuation of the auxin signaling, and inducer of senescence processes (Peng et al., 2021). Salicylic acid was also reported to mitigate abiotic stresses through activation of stress-induced genes, regulation of ion nutrition, involvement in redox signaling, enhancement of nitrate reductase activity or suppression of stress-induced chlorophyll degradation (Kaya et al., 2023; Kaur et al., 2022; Zhong et al., 2021; Liu et al., 2022). Under heavy metal stress, SA interacts with other plant growth regulators and promotes the stimulation of antioxidant compounds and enzymes to counteract stress (Sharma et al., 2020; Dar et al., 2023; Zaid et al., 2019; Li et al., 2023; Zheng et al., 2022).

Salicylic acid is synthesized by two alternative pathways (Fig. 1B). The first pathway occurs in cytoplasm and involves the conversion of phenylalanine to *trans*-cinnamic acid by phenylalanine ammonia lyase (PAL; EC 4.3.1.5). *Trans*-cinnamic acid is oxidized to benzoic acid which

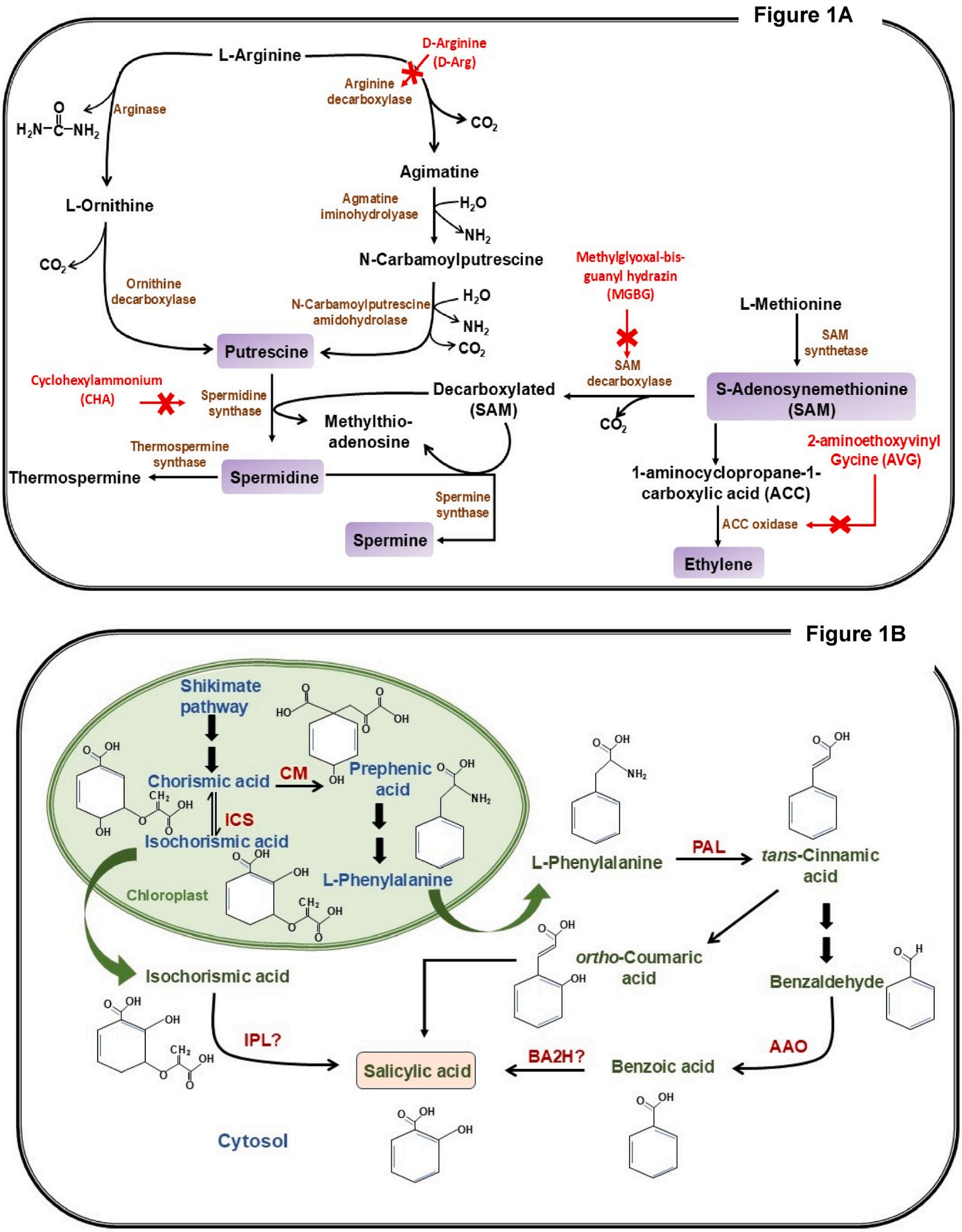

Fig. 1. Global overview of polyamines and ethylene biosynthetic pathways (A) and salicylic biosynthesis (B). The diamine putrescine is produced from arginine through arginine decarboxylase or from ornithine through ornithine decarboxylase. It is converted to the triamine spermidine by addition decarboxylated S-adenosylmethionine catalyzed by spermidine synthase and spermidine is converted to tetramine spermine by additional reaction with decarboxylated S-adenosylmethionine catalyzed by spermine synthase. Decarboxylated S-adenosylmethionine is produced by SAM decarboxylase from S-adenosylmethionine, which is also the precursor of 1-aminocyclopropane-1-carboxylic acid (ACC). ACC is then converted to ethylene by ACC oxidase. Inhibitors tested in the present study are indicated in red: D-Arginine (D-Arg) is an inhibitor of arginine decarboxylase cyclohexylammonium (CHA) is an inhibitor of spermidine synthase while methylglyoxal-bis-guanyl hydrazine (MGBG) is an inhibitor of SAM decarboxylase and 2-aminoethoxyvinyl glycine (AVG) is an inhibitor of ACC oxidase (A). Salicylic acid is produced in the chloroplast through conversion of chorismate to isochorismate by isochorismate synthase (ICS) with subsequent conversion of isochorismate to salicylic acid by isochorismte-pyruvate lyase (IPL). The second pathway occurs in cytosol and implies the conversion of phenylalanine to *trans*-cinnamic acid catalyzed by phenylalanine ammonia lyase (PAL). *Trans*-cinnamic acid is converted to benzoic acid which is then converted to salicylic acid by benzoic acid 2-hydroxylase (BA2H). Activities of ICS and PAL are considered as the rate limiting steps for the chloroplastic pathway and the cytosolic pathway, respectively (B).

is then hydroxylated to SA by benzoic acid 2-hydroxylase (EC 1.14.13). The second pathway, known as isochorismate (IC) pathway takes place in chloroplast and implies the conversion of chorismate to isochorismate by isochorismate synthase (ICS; EC 5.4.4.2) with subsequent conversion of IC to SA by isochorismate-pyruvate lyase (IPL; EC 4.2.99.21) (Peng et al., 2021; Zhong et al., 2021). Numerous studies provided experimental evidences that SA regulates polyamine synthesis (Gharbi et al., 2016; Pál et al., 2021). Indeed, exogenous SA was shown to regulate ADC gene (Canales et al., 2019; Takács et al., 2021; Rossi et al., 2021) and to decrease PAs degradation (Talaat, 2021). However, the reverse impact of PAs on SA biosynthesis was never evidenced until now.

To the best of our knowledge, no data are available regarding polyamine and salicylic putative involvement in Pb tolerance in *Piptatherum miliaceum*. Our tested hypothesis are that i) both polyamines and salicylic acid play a role in Pb tolerance of smilo grass and ii) these compounds exhibit specific interaction in Pb-stressed organs of *P. miliaceum*. In contrast to model plant species such as *Arabidopsis thaliana* or rice, the genome of *P. miliaceum* is not sequenced yet, and mutants for genes involved in PAs or SA synthesis or signaling are not available in this species. Moreover, no transgenic strategy is available for smilo grass. We thus took advantage of specific inhibitors of enzymes involved in the corresponding metabolic pathways and performed an exhaustive pharmacological approach to test our hypothesis.

2. Material and methods

2.1. Plant material and growth conditions

Seeds of *Piptatherum miliaceum* (L.) Cosson were harvested on plants growing in the mining district of La Union (SE Spain), at Cabezo de Don Juan near the city of Llano del Beal (37° 36′ 55″ N; 0° 50’ 10” W). Seeds were collected on 47 plants distributed on an area of 1500 m^2 and gathered to constitute one pooled sample. Five soil samples were collected on the first 15 cm, air dried and sieved at 2 mm. Soil samples were analyzed according to standard procedures (Page et al., 1982) with few modifications as detailed by Lambrechts et al. (2011). Total Pb concentration was up to 3373 ± 221 mg kg^{-1} DW. Detailed data of soil analyzes are provided in supplemental Table (Table S1).

Seeds were germinated in greenhouse conditions under 16 h photoperiod (mean light intensity of 230 μmol m^{-2} s^{-1}). Light was supplied by Philips HPIT 400-W lamps (Philips Lighting S.A., Brussels, Belgium) with a day/night temperature of 29/25 °C. Seeds were sown in a loam:silt substrate (1:1 v/v) regularly moistened with deionized water. Seedlings at the three leaves stages presenting uniform height (5.0 ± 0.5 cm) were transferred to a phytotron-growth chamber and distributed among 48 tanks (10 seedlings per tank) of 10 L half-strength Hoagland-modified nutrient solution containing (in mM) 5 KNO_3, 1 $NH_4H_2PO_4$, 0.5 $MgSO_4$, 5.5 $Ca(NO_3)_2$ and (in μM) 25 KCl, 10H_3BO_3, 1 $MnSO_4$, 1 $ZnSO_4$, 0.25 $CuSO_4$, 10 Na_2MoO_4 and 50 mg.L^{-1} FeEDTA. The solution pH was adjusted to 5.0 to help keeping Pb in its soluble form as recommended by Fischer et al. (2014). Plants were fixed on polystyrene plates at a mean distance of 6 cm. Temperature was 26 ± 1 °C during the day and 21 ± 1 °C during the night, with a mean PAR of 380 μmol m^{-2} s^{-1} provided by HPIT/400 W from PHILIPS metal iodide lamp and relative humidity was set at 50 ± 3 %. Solutions were renewed each week and were permanently aerated by an EHEIM compact ON300 pump. After 3 weeks of seedling growth in the phytotron, 500 μM $Pb(NO_3)_2$ was added to half of the tanks, in the presence or in the absence of salicylic acid 10 μM. Plants were harvested for subsequent analysis after 6 weeks of treatment. According to the Visual MINTEQ speciation software, the vast majority of added Pb remained in the solution as Pb^{2+} (85.19 %), with minor proportion of $PbSO_4$(aq) (7.49 %), $PbNO_3^+$ (4.94 %) and $PbH_2PO_4^+$ (1.76 %). The remaining part ($(Pb(OH)^+$, $PbCl^+$, $PbHPO_4$ (aq)) constituted less than 0.6 %.

At the same time, and in another set of plants, the following metabolic inhibitors of PAs and ethylene pathways were tested (Fig. 1): D-arginine 5 mM (D-Arg; inhibitor of arginine decarboxylase), methylglyoxal-bis-guanyl hydrazone 1 mM (MGBG; inhibitor of S-adenosylmethionine decarboxylase), cyclohexylammonium 5 mM (CHA; inhibitor of spermidine synthase), 2-aminoethoxyvinyl glycine 2 μM AVG (inhibitor of aminocyclopropane carboxylic acid (ACC) oxidase involved in ethylene synthesis; EC 1.14.17.4)). The impact of each water-soluble inhibitor was tested separately, and inhibitors were added concomitantly to $Pb(NO_3)_2$. Doses of inhibitors were established based on previous experiments (Ndayiragije and Lutts, 2006; Zhou et al., 2018). D-Arg, CHA and AVG were purchased from Sigma-Aldrich/Merck-Belgium while MGBG was obtained from MCE (MedChemExpress; Belgium). Purity of the products was >99 % for AVG and CHA, >98 % for D-Arg, and 99.13 % for MGBG. Additional treatments also required exogenous application of putrescine, spermidine and spermine 100 μM or salicylic acid 10 μM (Sigma-Aldrich/Merck-Belgium) as stated in the text. For each treatment, 3 tanks of 10 plants were thus considered and randomly disposed in the phytotrons.

2.2. Root and shoot ion concentration after 6 weeks of treatment

The roots and shoots of six plants per treatment were considered: roots and shoot of each plant were separately incubated in an oven at 72 °C during 48 h until reaching constant dry weight. Each organ was then ground to obtain a homogeneous fine powder. Samples of dry matter (*c.a.* 50 mg) were digested by incubation in a mix of 68 % HNO_3 and 37 % HCl at 80 °C on a sand bath. After complete evaporation, minerals were re-dissolved in HCl 0.1 N and filtered on a Whatman n°1 filter paper. Elements were quantified by ICP-OES inductively plasma emission spectrometry (Thermo Jarrel Ash Iris Advantage). Samples were injected through a Mainhard nebulizer and carried through an argon gas plasma condition with reflected power of 7 W and forward power of 1300 W. Gas flow rates of plasma, auxiliary, and nebulizer were set at 16.0, 1.0 and 1.0 L min^{-1}, respectively. The internal standard multi-element stock solution obtained from Agilent (USA) allowed us to control stability of the instrument while certified reference material ERM-CA713 was obtained from Sigma-Aldrich (Germany).

2.3. Salicylic acid, polyamines and ethylene quantification

Salicylic acid quantification in roots and shoots was conducted on 200 mg FW; 1.8 mL HCl 1 M was added and incubation was conducted during 3 h under mechanical shaking (100 rpm) at room temperature before sonication of the mixture during 1 min at 5 °C. The homogenate was then centrifuged during 15 min at 15,000 *g* at 5 °C. The ethyl acetate fractions were evaporated to dryness at 45 °C on a speedvac. Residues were dissolved in 0.5 mL water/acetonitrile (ACN) (1:1 v/v) according to Molinari and Loffredo (2006). Salicylic acid quantification was conducted with an Agilent 1260 series HPLC on 5 μL of injected samples. The used column was an Inertsil ODS-3 (250 × 3.0 mm, 3 μm) thermostated at 30 °C. The mobile phase consisted in a water/ACN gradient from 10 to 100 % ACN and the flow was set at 1.0 mL min^{-1}. Salicylic acid was detected by a fluorescence detector at a 315 nm emission and a 408 nm excitation wavelength. Quantification of SA was performed by external calibration using SA standards (SigmaChemicals) with concentrations from 0.78 to 100 μM.

Total phenolic concentration was assayed using the Folin-Ciocalteu reagent, following Singleton's method (Singleton and Rossi, 1965) with slight modifications. An aliquot (20 μL) of sample was added to 1.58 mL of deionized water and 100 μL of the raw Folin-Ciocalteu reagent. The mixture was shaken and allowed to stand for 6 min before adding 300 μL of 2 % sodium carbonate (Na_2CO_3) solution. After incubation for 2 h at room temperature in the dark, the absorbance was measured at 765 nm. Total phenolic contents were expressed as milligrams of gallic acid equivalents per gram of fresh weight (mg of GAE g^{-1} FW) through calibration curve with gallic acid.

For free PAs determination 250 and 500 mg FW for roots and shoots, respectively were extracted twice with 4 % $HClO_4$ (v/v) at 4 °C and derivatized by dansylation according to Quinet et al. (2014). Samples were resuspended in methanol and filtered (Chromafil PES-45/15, 0.45 µm; Macherey-Nagel, Düren, Germany). Analyses were conducted by a Shimadzu HPLC system coupled to a RF-20A fluorescence detector (Shimadzu,'s-Hertogenbosch). Detection was performed using an excitation wavelength of 340 nm and an emission wavelength of 510 nm. The used column was a Nucleodur C_{18} Pyramid column (125 × 4.6 mm internal diameter, 5 µm particle size; Macherey-Nagel) thermostated in an oven at 40 °C. The mobile phase consisted of a water/ACN gradient from 40 to 100 % ACN with a flow of 1.0 mL min^{-1}. To prepare the reference standard stock solution, polyamines (Put, Spd, Spm (Sigma-Aldrich; Belgium)) were dissolved in methanol at a concentration of 1000 µg mL^{-1}. Working solutions were prepared using serial dilution with methanol at concentrations ranging from 1 to 100 µg mL^{-1}. The internal standard consisted in 1,7-diaminoheptane dissolved in methanol at 100 µg mL^{-1}. All solutions were kept at −20 °C until use. The QC samples were prepared from a different set of working stock than the calibration solution. Calibration curves were plotted with concentrations ranging from 1 to 5000 ng mL^{-1} and the linearity was evaluated using the correlation coefficient (r^2) of the calibration curve.

An ethylene detector ETD-300 (Sensor Sense, Nijmegen, The Netherlands) was used for ethylene determination. For each plant, four leaves located at the middle part of the main stem were introduced in glass dishes with two layers of filter paper moistened with 5 mL of sterile deionized water. The measurements were conducted in a growth chamber (16 h photoperiod, 200 µmol m^{-2} s^{-1} irradiance, 22 °C) in a stop-and-flow mode with each cuvette being alternatively flushed with a flow of 3 L h^{-1} during 22 min according to Cristescu et al. (2002). An empty dish without leaves was used as control.

2.4. Enzyme activities

Phenylalanine ammonia-lyase (PAL; EC4.3.1.5) activity was measured as previously described by Solecka and Kacperska (2003) with slight modification. Briefly 2 g of frozen fresh leaves were homogenized with 12 mL of extraction buffer containing 50 mM Tris-HCl buffer (pH 8.9), 15 mM β-mercaptoethanol, 5 mM ethylenediamine tetra-acetic acid (EDTA), 5 mM ascorbic acid, 10 mM leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 0.15 % (w/v) polyvinyl pyrrolidone (PVP). The homogenate was filtered through 4 layers of cheesecloth and centrifuged at 12,000 *g* for 20 min at 4 °C. The supernatant was then used as a source of crude enzymes for assaying PAL activity. The reaction mixture (3 mL) contained 16 mM L-phenylalanine, 50 mM Tris-HCl buffer (pH 8.9), 3.6 mM NaCl and 0.5 mL crude enzyme. The substrate was pre-incubated at 37 °C for 15 min before mixing with the enzyme extract. The reaction was performed at 37 °C for 1 h and was stopped by adding 500 µL 6 M HCl. The tubes were then centrifuged for 10 min at 12,000 *g* to pellet the denatured protein. The absorbance was measured at 290 nm against a blank without substrate. PAL activity equaled the amount of PAL that produced 1 µg of cinnamic acid (CA) in 1 h, and was expressed as µg CA h^{-1} mg^{-1} protein. Protein concentrations were determined according to Bradford (1976).

Isochorismate synthase (ICS; EC 5.4.4.2) activities were determined as detailed by Poulsen et al. (1991) with slight modifications recommended by Nugroho et al. (2001). Briefly, frozen samples were ground and 1.5 g FW was added with 0.05 g PVPP and 2 mL of extraction buffer consisting in 0.1 M Tris-HCl pH 7.5, 1 mM EDTA, 1 mM DTT and 10 % glycerol. The homogenate was then centrifuged at 10,000 *g* and 4 °C during 20 min. The recovered supernatant was desalted at 4 °C on Sephadex G-25 column equilibrated with 0.1 M Tris-HCl containing 10 % glycerol. Reaction was performed during 1 h at 30 °C in a total volume of 1 mL with 500 µL of 0.1 M Tris-HCl pH 7.5 containing 3 mM Ba-chorismate, 15 mM $MgCl_2$ and 500 µL of enzyme extracts. Produced isochorismate was quantified according to Seeger and Bentley (1991) through an HPLC system at room temperature on a 4.0 mm × 250 mm LiChorsorb Select B column with a particle size of 7 µm at a flow rate of 1 mL min^{-1}. The eluent consisted in 50 mM H_3PO_4 in H_2O–MeOH (13:6) and 6 M NaOH was used to adjust pH to 2.7 in the water phase before addition of MeOH. Absorbance was monitored at 280 nm.

2.5. Cell viability index and cell membrane stability

Cell viability was determined on leaf segments (*c.a.* 50 mg FW; leaves collected at the middle part of the main stem) quickly rinsed in deionized water containing 0.05 % Tween-20 and incubated in darkness at 30 °C in glass tubes containing 5 mL of 0.5 % triphenyltetrazolium chloride (TTC; Sigma-Chemical) dissolved in 50 mM K_2HPO_4 at pH 7.0 during 15 h. Similar procedure was used on root samples collected at the top 2 cm of the roots. Samples recovered after filtration were then incubated for 5 min in 3 mL glass vials containing 94 % ethanol at 80 °C. The extracted formazan recovered after centrifugation at 5000 *g* was quantified spectrophotometrically at 487 nm, and viability index was defined as absorbance measured per g FW according to Lutts et al. (2004). Cell membrane stability was determined on the same plants using the electrolyte leakage method as previously described (Bajji et al., 2002) and expressed as the electrolyte leakage ratio (EL; in %).

2.6. Malondialdehyde and total antioxidant power

Oxidative stress was quantified by estimating malondialdehyde (MDA) as a by-product of lipid peroxidation in roots and leaves of six plants per treatment, following the method of Heath and Packer (1968). Frozen samples (0.25 g FW) were homogenized in a prechilled mortar with 10 mL of ice-cold 5 % (w/v) trichloroacetic acid and centrifuged at 12,000 *g* for 10 min at 4 °C. An assay mixture containing a 2 mL aliquot of supernatant and 2 mL of 0.67 % (w/v) thiobarbituric acid was heated to 100 °C for 30 min and then rapidly cooled to 4 °C in an ice bath. After centrifugation at 12,000 *g* for 1 min at 4 °C, the supernatant absorbance was recorded (532 nm), and values corresponding to nonspecific absorption (600 nm) were subtracted. Malondialdehyde concentration was calculated using its molar extinction coefficient (155 mM cm^{-1}).

The estimation of the total antioxidant capacity of the samples was based on the reduction of 2,2-diphenyl-1-picrylhydrazyl (DPPH) according to Chen et al. (1999) with some adaptations. For each sample, 100 µM of the methanolic extract was mixed with 3 mL of 0.06 mM DPPH in methanol. After incubation in darkness (30 min), the absorbance at 517 nm was measured against methanol blank. The scavenging percentage of DPPH radical was calculated according to Maisuthisakul et al. (2007): DPPH radical scavenging capacity (%) = $[A_0-(A_1-A_S)]/A_0 \times 100$ where A_0 was the absorbance of the control solution (containing only DPPH), A_1 was the absorbance for the plant extract in the presence of the DPPH solution, and A_S was the absorbance of the sample extract solution without DPPH, which was used for correcting the unequal color of the sample extracts. Each sample was analyzed by triplicate.

2.7. Glutathione and ascorbate quantification

Total (GSHt) and reduced (GSH) glutathione were determined according to Cereser et al. (2001) after extraction and derivatization by orthophthalaldehyde (OPA). Dithiotreitol (DTT) was used to reduce oxidized glutathione (GSSG). Filtration of the extracts was performed with a Chromafil PES-45/15 0.45 µm microfilters (Macherey-Nagel). Five µL of sample were injected into a Shimadzu HPLC system (Shimadzu, 's-Hertogenbosch) and OPA derivatives were separated on a reversed-phase column (Nucleodur C18 Pyramid column (125 × 4.6 mm internal diameter; 5 µm particle size; Macherey-Nagel)) using an acetonitrile-sodium acetate gradient in a 50 mM sodium acetate buffer pH 6.2 at 30 °C at a flow rate of 0.7 mL min^{-1}. Detection was performed with a spectra system Shimadzu RF-20A fluorescence detector at 420 nm after excitation at 340 nm. A nine-point calibration curve using external GSH standard solution (from 0.0625 to 50 μM) was used to calibrate the system and the GSH recovery was determined using GSH as an internal standard.

Ascorbate quantification was performed according to Kampfenkel et al. (1995). A 0.25 g sample of leaf tissue was homogenized with 4 mL of trichloroacetic acid (TCA) and left for 15 min on ice. The samples were then centrifuged for 5 min at 10,000 *g* at 4 °C and the supernatant was recovered. To determine total ascorbate, oxidized ascorbate (DHA) was reduced to ascorbic acid (AsA) with DTT and excess DTT was removed with NEM. For the determination of reduced ascorbate, 0.2 mL of the supernatant, 0.4 mL of 0.2 M phosphate buffer pH 7.4, 0.4 mL of miliq water, 1 mL of 10 % w/v TCA, 0.8 mL of 85 % H_3PO_4, 0.8 mL of 4 % dipyridyl and 0.4 mL of 3 % $FeCl_3$ were used. The solutions to determine reduced ascorbate and total ascorbate were incubated for 1 h at 37 °C and the absorbance was read in spectrophotometer at a wavelength of 525 nm. The determination of oxidized ascorbate (DHA) was estimated by subtracting the total minus reduced ascorbate values.

2.8. *Non-protein thiol and phytochelatin estimation*

For total non-protein thiols (NPT) determination, 200 mg FW was ground at 0 °C in a pre-chilled mortar in 2 mL of 5 % (*w/v*) sulfosalicylic acid plus 6.3 mM diethylenetriaminepentaacetic acid (pH < 1). Samples were centrifuged at 4 °C during 10 min at 10,000 *g*. Thiols were determined in the supernatant using Ellman's reagent according to De Vos et al. (1992): 300 μL were mixed with 630 μL of 0.5 M KH_2PO_4 and 25 μL of 10 mM 5,5-dithiobis 2-nitrobenzoic acid (final pH 7.0). The absorbance was recorded at 412 nm after 2 min incubation at room temperature, and the NPT concentration was estimated using an extinction coefficient of 13,600 M^{-1} cm^{-1}. As recommended by Schäfer et al. (1997) phytochelatins content was evaluated as difference between NPT and total glutathione concentrations.

2.9. *Statistical analysis*

Each treatment was applied to 3 tanks of 10 plants. Tanks were distributed in the phytotron according to a complete randomized design. Morphological parameters and ion content were estimated on 6 plants per treatment. The 24 remaining plants were assembled in 8 lots of three pooled plants. Four lots were considered for PAL and ICS activities, ethylene estimation, viability index and electrolyte leakage and the remaining four lots were used for metabolites quantification (SA, PAs, MDA, GSH, ascorbate, phytochelatins) and total antioxidant power. For a given sample, each measurement was performed with technical triplicates. The statistical analysis was carried out using Statistica v.5.0 (Statsoft. Inc.). Normality of the data was estimated using Shapiro-Wilk tests, and homoscedasticity using the Levene test. Percentage data were arc-sin transformed before analysis. One-way analysis of variance (ANOVA) was used to determine the main effects of the treatments on the physiological and biochemical parameters on the studied plants of *Piptatherum milliaceum*. The Tukey HSD test at the 0.05, 0.01 and 0.001 probability level, was used to determine the significance of the differences. Correlation analysis were performed separately for shoots and roots on data pooled from all 13 treatments considering the four biological replicates leading to 52 data sets. The Pearson correlation coefficient was calculated as the ratio between the covariance of each pair of variables and the product of their standard deviations. The experiment was repeated three times and gave similar trends.

3. Results

3.1. *Lead impact on growth and physiological properties of Piptatherum miliaceum*

All plants, including stressed ones, remained alive until the end of the treatment. Lead exposure induced a significant decrease in both shoots and roots fresh and dry weights (Table 1) but had no impact on tiller number. It decreased water content in the shoots but not in the roots. Lead accumulated to a higher extent in the roots than in the shoots since Pb concentration was more than 10 times higher in the former than in the latter (Table 1). Lead induced a slight decrease in shoot Cu, Mg, Ni and Mn concentration together with a slight, although significant, decrease in root Ca and Fe concentrations (Table S2). In contrast, S concentration increased by 71 and 42 % in roots and shoots of plants exposed to Pb comparatively to control plants (Table S2). Lead accumulation significantly reduced cell viability estimated on the basis of TTC test in shoots and roots and increased electrolyte leakage in shoots only (Table 1). Concentration of all PAs (Put, Spd, Spm) and ethylene synthesis increased in the shoot and roots of plants exposed to Pb toxicity.

In the absence of stress, SA and total phenolics concentrations were higher in shoots than in roots (Table 1). Lead obviously increased SA and total phenolics in both organs. Phenylalanine ammonia-lyase activities

Table 1

Impact of lead treatment on morphological, physiological and biochemical properties of *Piptatherum miliaceum*. Stressed plants were exposed to 500 μM Pb in nutrient solution during 6 weeks. Each value is the mean of 6 replicates ± S.E. for morphological parameters weight, number of tillers and Pb content and the mean of 4 biological replicates (3 pooled plant per replicate) for biochemical parameters. For a given organ and parameter, means followed by different letters are significantly different at $P < 0.05$ according to Tukey HSD test. (WC: water content; TTC: triphenyltetrazolium chloride; Put: putrescine; Spd: spermidine; Spm: spermine; AsA: ascorbic acid; DHA: oxidized ascorbate; DPPH: 2,2-diphenyl-1-picrylhydrazyl; GSH: reduced glutathione; GSSG: oxidized glutathione; MDA: malondialdehyde; PC: phytochelatin). ND: not detected.

Parameter	Shoots		Roots	
	Control	Pb	Control	Pb
Fresh weight	34.1 ± 0.32 a	16.5 ± 1.93 b	18.4 ± 0.56 a	11.7 ± 1.23 b
Dry weight (g)	5.73 ± 0.48 a	3.57 ± 0.45 b	2.13 ± 0.07 a	1.48 ± 0.15 b
Number tillers	17.8 ± 1.4 a	16.9 ± 1.9 a	–	–
WC (%)	83.2 ± 0.9 a	78.4 ± 1.0 b	88.4 ± 1.2 a	87.3 ± 1.9 a
Pb (mg.Kg^{-1})	1.4 ± 0.4 a	84.2 ± 4.1 b	2.3 ± 0.6 a	901.6 ± 30.8 b
TTC (DO 487 nm. g^{-1}FW)	0.67 ± 0.05 a	0.41 ± 0.09 b	0.56 ± 0.10 a	0.21 ± 0.08 b
EL (%)	8.6 ± 0.7 a	19.7 ± 1.3 b	14.9 ± 0.8 a	15.2 ± 1.3 a
Put (μmol.g^{-1} FW)	71.3 ± 6.4 a	121.4 ± 11.0 b	111.1 ± 9.1 a	147.9 ± 7.4 b
Spd (μmol.g^{-1} FW)	65.2 ± 3.5 a	80.9 ± 4.1 b	88.4 ± 5.4 a	118.4 ± 9.4 b
Spm (μmol.g^{-1} FW)	15.6 ± 1.21 a	63.2 ± 3.2 b	25.7 ± 2.6 a	66.9 ± 6.7 b
Ethylene (nL.h^{-1} g^{-1} FW)	1.14 ± 0.22 a	2.95 ± 0.05 b	0.58 ± 0.17 a	2.11 ± 0.12 b
SA (μg g^{-1} FW)	64.2 ± 5.4 a	136.8 ± 10.4 b	45.1 ± 3.9 a	117.2 ± 11.2 b
Total phenolics	20.61 ± 2.14 a	41.32 ± 2.84 b	10.74 ± 1.34 a	31.2 ± 2.21 b
PAL (μg h^{-1} mg^{-1} prot)	19.7 ± 1.17 a	56.5 ± 4.02 b	18.3 ± 11.34 a	38.5 ± 6.3 b
ICS (ng h^{-1} mg^{-1} prot)	7.54 ± 1.7 a	20.7 ± 4.51 b	ND	2.32 ± 0.98
MDA (nmol.g^{-1} DW)	9.8 ± 0.6 a	22.4 ± 1.1 b	8.4 ± 0.5 a	34.7 ± 2.9 b
DPPH (%)	45.8 ± 3.1 a	63.9 ± 1.7 b	38.4 ± 2.4 a	71.5 ± 4.1 b
GSH (nmol.g^{-1} FW)	226 ± 13 a	375 ± 28 b	118 ± 19 a	258 ± 31 b
GSSG (nmol.g^{-1} FW)	13 ± 2 a	197 ± 12 b	15 ± 1 a	205 ± 17 b
AsA (nmol.g^{-1} FW)	237 ± 23 a	398 ± 41 b	101 ± 15 a	113 ± 14 a
DHA (nmol g^{-1}FW)	25 ± 3 a	89 ± 6 b	45 ± 3 a	74 ± 8 b
PC (nmol.g^{-1} FW)	24 ± 7 a	111 ± 22 b	33 ± 8 a	357 ± 41 b

were similar in shoots and roots of control plants (Table 1); Pb increased PAL activity to a higher extent in the shoots than in the roots. Isochorismate synthase activity also increased in response to Pb in the shoots but ICS activity remained undetectable in the roots of control plants and exhibited rather low values in the roots of stressed ones (Table 1).

Malondialdehyde and total antioxidant power (Table 1) increased to similar extent in roots and shoots of plants exposed to Pb toxicity comparatively to controls. Reduced glutathione (GSH; Table 1) also increased in response to Pb stress but reached higher levels in the shoots than in the roots. In contrast, GSSG which also increased in stressed plants, accumulated to similar values in the two types of organs. The GSH/GSSG ratio was 17.4 and 7.9 in the shoots and roots of control plants, respectively, but it decreased to 1.9 in the shoots and to 1.3 in the roots of stressed plants. Ascorbic acid (Table 1) increased in response to Pb in the shoots but not in the roots of stressed plants while DHA increased in both types of organs. Lead induced a strong increase in phytochelatins (PC) concentration in the roots and a lower increase in the shoots.

3.2. Impact of inhibitors of polyamine synthesis and exogenous salicylic acid on plant response to Pb toxicity

The impacts of inhibitors and exogenous SA on non-stressed plants are detailed in Table S3 (supplemental data). When plants exposed to 500 µM Pb were cultivated in the presence of D-Arg, all polyamine concentrations decreased in shoots and roots (Table 2). However, supplying Put simultaneously to D-Arg allowed to recover a polyamine concentration similar (shoot) or even higher (roots) than plants not exposed to inhibitors. Lead-treated plants exposed to CHA exhibited an increase in Put but a significant decrease in Spd and Spm. Once again, supplying Spd simultaneously with CHA allowed to recover normal Spd and Spm titers. A similar trend was recorded in non-stressed plants (Table S3) confirming the efficiency of inhibitors in both conditions. It is of special interest to notice that in Pb-treated plants (Table 2), these inhibitors decreased SA content in the shoots but not in the roots. Supplying exogenous polyamine restored the SA content in the shoots. However, supplying CHA + Put decreased Spd and Spm in all organs, as well as SA content in the shoots.

Table 2

Concentration of putrescine (Put), spermidine (Spd), spermine (Spm) and salicylic acid (SA) in roots and shoots of *Piptatherum miliaceum* cultivated during 6 weeks in the presence of 500 µM Pb, and exposed to D-Arginine (D-Arg; 5 mM), cyclohexylammonium (CHA; 5 mM), exogenous putrescine (Put; 100 µM), exogenous spermidine (Spd; 100 µM)) or different combinations of inhibitors and exogenous polyamines. Concentrations of PAs are provided in µmol g^{-1} FW and concentration of SA in µg g^{-1} FW. Each value is the mean of 4 biological replicates (3 pooled plant per replicate) ± S.E. For a given organ and a given parameter, values followed by different letters are significantly different at $P < 0.05$ according to the Tukey HSD test.

Treatment	Shoots				Roots			
	Put (µmol g^{-1} FW)	Spd	Spm	SA (µg g^{-1} FW)	Put (µmol.g^{-1} FW)	Spd	Spm	SA (µg. g^{-1} FW)
Pb	118 ± 7 b	93 ± 7 bc	64 ± 4 c	138 ± 11 b	122 ± 10 b	79 ± 6 c	37 ± 4 c	101 ± 8 a
Pb + D-Arg	61 ± 4 a	42 ± 5 a	35 ± 3 ab	77 ± 5 a	71 ± 4 a	28 ± 4 a	14 ± 2 a	92 ± 12 a
Pb + D-Arg + Put	131 ± 13 bc	107 ± 11 c	71 ± 6 c	127 ± 12 b	158 ± 11 c	123 ± 8 d	48 ± 5 c	97 ± 7 a
Pb + CHA	169 ± 11 c	38 ± 6 a	27 ± 2 a	63 ± 4 a	174 ± 9 c	32 ± 4 ab	15 ± 3 ab	89 ± 14 a
Pb + CHA + Spd	175 ± 9 c	88 ± 9 b	71 ± 6 c	121 ± 9 b	163 ± 10 c	85 ± 6 c	47 ± 4 c	97 ± 12 a
Pb + CHA + Put	313 ± 32 d	45 ± 4 a	40 ± 4 b	70 ± 4 a	305 ± 11 d	37 ± 5 b	20 ± 4 b	106 ± 15 a

Fig. 2 presents the dry weight, Pb concentrations, cell viability (TTC test) and cell membrane stability (electrolyte leakage) in shoots and roots of plants exposed to 500 µM Pb and to inhibitors of polyamine synthesis in the presence or in the absence of exogenous polyamines or SA treatment. Application of D-Arg or CHA decreased shoot and root DW, the recorded decrease being more important at the shoot level (Fig. 2A) than in the roots (Fig. 2B). Supplying exogenous Put to D-Arg treated plants or Spd to CHA-exposed ones restored similar dry weights than untreated plants exposed to Pb only. However, supplying exogenous Put to CHA-treated plants strongly decreased dry weights. Exposure of plants to exogenous SA 10 µM did not improve shoot and root DW in plants exposed to Pb in the absence of inhibitors. In contrast, exogenous SA improved the shoot DW of plants simultaneously exposed to D-Arg or CHA. The considered treatments had no impact on the water content (detailed data not shown). A significant increase in the shoot Pb concentration (Fig. 2C) as well as a decrease in the root Pb concentration (Fig. 2D) were recorded for plants exposed to D-Arg or CHA but the recorded effect was cancelled by exogenous application of Put or Spd, respectively. This was not any more the case when Put was supplied to CHA-treated plants. Supplying additional SA had no significant effect on shoot and root Pb concentrations. Both tested inhibitors compromised cell viability estimated by TTC reduction to a similar extent in the leaves (Fig. 2E) and in the roots (Fig. 2F). Exogenous Put and Spd only partly mitigated this deleterious impact of D-Arg and CHA on Pb-treated plants in the shoots while exogenous SA induced the highest improvement of cell viability index in the absence or in the presence of inhibitors. A reverse situation was observed for cell membrane stability (Fig. 2G and H): EL indeed increased in Pb-treated plants exposed to D-Arg and CHA, especially at the root level (Fig. 3H). Such an increase was strongly minimized by exogenous Put and Spd while exogenous SA only marginally modified EL values. Inhibitors of PAs synthesis also reduced cell viability and plant growth in non-stressed plants but their impacts were from a relative point of view by far lower than in Pb-treated plants (Table S3).

Activities of PAL and ICS are provided by Table 3. At the shoot level, PAL activity was not affected by D-Arg but was significantly reduced by CHA. Supplying Spd to CHA-treated plants allowed to overcome the recorded inhibitor effect but exogenous Put did not. Exogenous SA also slightly increased PAL activities in the shoot. Phenylalanine ammonia lyase activity was lower in the roots than in the shoots. It remained unaffected by inhibitors of PAs synthesis but slightly increased in response to SA treatment. Isochorismate synthase displayed a contrasting behavior comparatively to PAL. At the shoot level, it was strongly inhibited by D-Arg and CHA and only exogenous PAs allowed to recover values close to plants exposed to Pb stress in the absence of inhibitors with the noticeable exception of Put applied on CHA-treated plants. Root ICS activity was quite low in Pb-treated plants. No activity was detected in the presence of D-Arg or CHA, although additional Put in the first case and Spd in the second one allowed a modest recovery. In the shoots of Pb-treated plants, both phenolic compounds concentrations and DPPH (Table 3) were affected by D-Arg and exhibited the lowest value in plants exposed to CHA. Exogenous SA and Put allowed a partial recovery for these parameters in D-Arg treated plants while exogenous SA and Spd similarly mitigated the deleterious impact of CHA on plants exposed to Pb 500 µM. Such effects, however, were not recorded in plants not exposed to Pb (Table S3). Exogenous Put did not significantly increase phenolic compounds and DPPH in the roots of D-Arg treated plants. The highest DPPH value in roots of Pb-stressed plants was recorded for plants exposed to CHA + Spd treatment. Exogenous SA in the absence as well as in the presence of PAs inhibitors allowed to recover phenolics and DPPH values close to the Pb-treated control plants.

Shoot and root MDA (Fig. 3A and B) exhibited similar trends: MDA

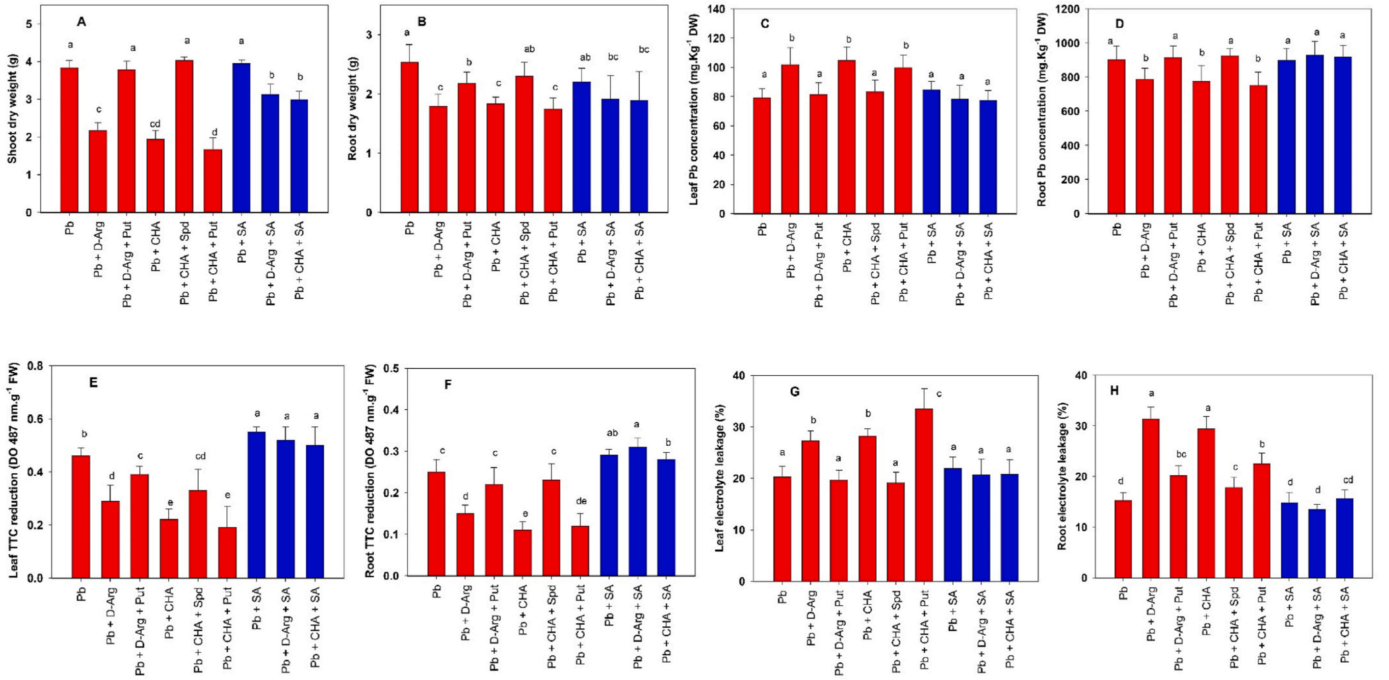

Fig. 2. Dry weights (in g; A and B), Pb concentrations (in mg kg^{-1} DW; C and D), cell viability index (TTC reduction, in DO 487 nm g^{-1} FW; E and F) and electrolyte leakage (in %; G and H) in shoots (A, C, E and G) and roots (B, D, F and H) of *Piptatherum miliaceum* cultivated during 6 weeks in the presence of Pb 500 μM. Plants were simultaneously exposed to D-Arginine (D-Arg, 5 mM) or cyclohexylammonium (CHA; 5 mM) in the absence or in the presence of supplemental exogenous putrescine (Put 100 μM), exogenous spermidine (Spd 100 μM) or exogenous salicylic acid (SA, 10 μM). For dry weight and ion content, each value is the mean of 6 replicates. For cell viability and electrolyte leakage, each value is the mean of 4 biological replicates (each replicate comprising three pooled plants). Vertical bars are standard errors. Values with different letters are significantly different at $P < 0.05$ according to the Tukey HSD test. Note that vertical scales are not necessarily the same for shoots and roots.

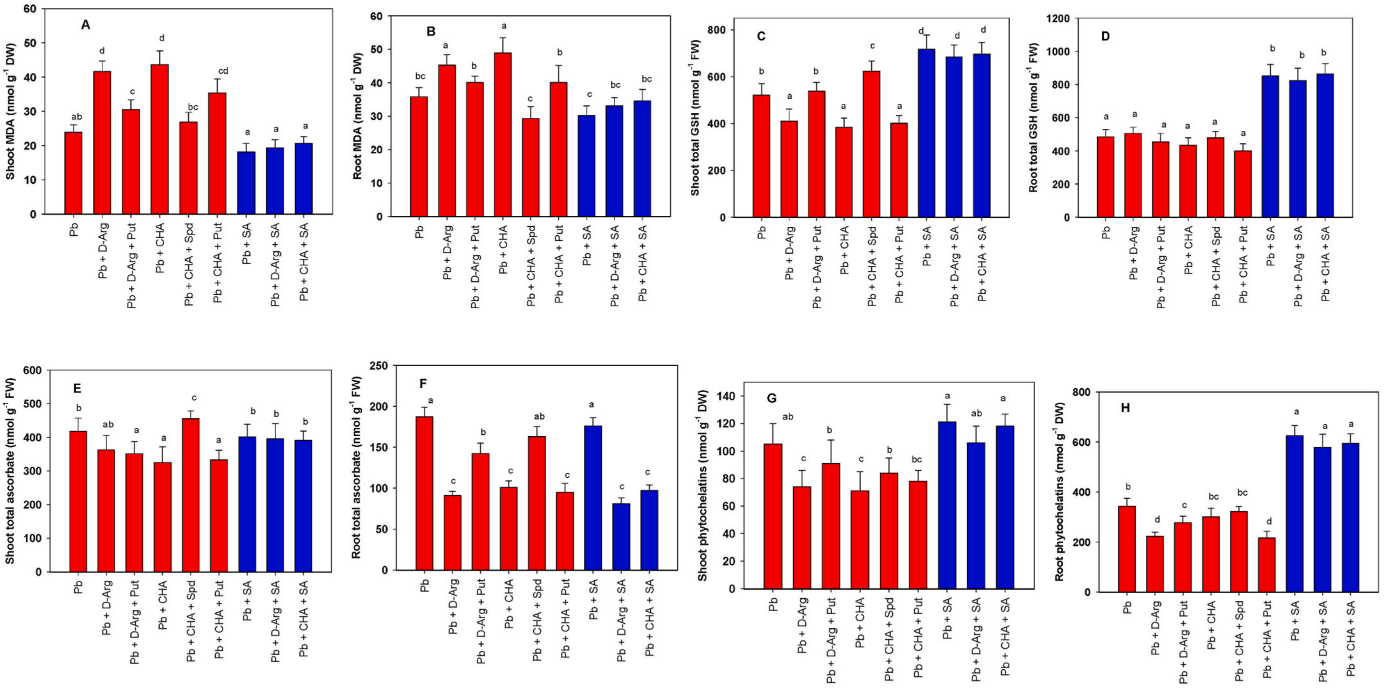

Fig. 3. Malondialdehyde (MDA; in nmol.g^{-1} DW; A and B), total glutathione (in nmol.g^{-1} FW; C and D), total ascorbate (in nmol g^{-1} FW; E and F) and total phytochelatins (in nmol g^{-1} FW G and H) concentrations in shoots (A, C, E and G) and roots (B, D, F and H) of *Piptatherum miliaceum* cultivated during 6 weeks in the presence of Pb 500 μM. Plants were simultaneously exposed to D-Arginine (D-Arg, 5 mM) or cyclohexylammonium (CHA; 5 mM) in the absence or in the presence of supplemental exogenous putrescine (Put 100 μM), exogenous spermidine (Spd 100 μM) or exogenous salicylic acid (SA, 10 μM). Each value is the mean of 4 biological replicates (each replicate comprising three pooled plants) and vertical bars are standard errors. Values with different letters are significantly different at $P < 0.05$ according to the Tukey HSD test. Note that vertical scales are not necessarily the same for shoots and roots.

Table 3
Phenylalanine ammonia lyase activity (PAL; in μg cinnamic acid.h^{-1} mg^{-1} prot), isochorismate synthase activity (ICS; in ng chorismate.h^{-1} mg^{-1} prot.), total phenolic compound concentration (mg gallic acid equivalent (GAE).g^{-1} FW), and total antioxidant power (DPPH; in %) in shoots and roots of *Piptatherum miliaceum* cultivated during 6 weeks in the presence of 500 μM Pb, and exposed to ᴅ-Arginine (D-Arg; 5 mM)), cyclohexylammonium (CHA; 5 mM), exogenous putrescine (Put; 100 μM)), exogenous spermidine (Spd; 100 μM) or exogenous salicylic acid (SA; 10 μM) in different combinations. Each value is the mean of the mean of 4 biological replicates (3 pooled plant per replicate) ± S.E. For a given organ and a given parameter, values followed by different letters are significantly different at $P < 0.05$ according to the Tukey HSD test. ND: not detected.

Treatment	Shoots				Roots			
	PAL(μg CA h^{-1} mg^{-1} prot)	ICS(ng.h^{-1} mg^{-1} prot)	Phenolics (mg GAE g^{-1} FW)	DPPH (in %)	PAL (μg CA h^{-1} mg^{-1} prot)	ICS (ng.h^{-1}mg^{-1} prot)	Phenolics (mg GAE g^{-1} FW)	DPPH (in %)
Pb	53.1 ± 4.7 b	19.7 ± 1.2 c	38.3 ± 2.1 a	72.8 ± 4.5 a	26.0 ± 2.9 a	2.5 ± 0.3 a	27.2 ± ab	68.3 ± 1.4 b
Pb + D-Arg	48.2 ± 4.3 b	10.2 ± b	29.2 ± 1.7 c	59.1 ± 2.6 c	24.9 ± 1.8 a	ND	20.1 ± c	50.2 ± 2.6 d
Pb + D-Arg + Put	57.1 ± 6.1 bc	18.3 ± 0.9 c	35.6 ± 2.3 ab	64.5 ± 3.0 b	23.5 ± 2.0 a	2.8 ± 0.2 a	23.4 ± bc	55.6 ± 3.4cd
Pb + CHA	40.9 ± 3.7 a	6.1 ± 0.5 a	21.4 ± 0.7 e	55.2 ± 1.4 d	27.3 ± 2.2 a	ND	19.5 ± cd	47.7 ± 3.9 d
Pb + CHA + Spd	56.3 ± 5.0 c	15.2 ± 3.4 bc	25.1 ± 0.4 d	66.9 ± 5.7 b	23.6 ± 3.1 a	2.3 ± 0.5 a	25.1 ± b	76.1 ± 1.3 a
Pb + CHA + Put	42.4 ± 4.1 ab	9.8 ± 1.7 b	20.3 ± 0.7 e	58.3 ± 3.1 c	24.7 ± 1.5 a	ND	18.1 ± d	62.5 ± 2.2 c
Pb + SA	54.5 ± 5.2 bc	19.4 ± 1.1 c	40.9 ± 3.1 a	75.1 ± 1.3 a	35.8 ± 3.1 b	ND	30.6 ± a	70.4 ± 10 b
Pb + D-Arg + SA	62.8 ± 4.9 c	9.3 ± 1.0 ab	34.1 ± 2.7 b	69.1 ± 2.3 ab	36.4 ± 2.8 b	ND	31.4 ± a	66.8 ± 3.7 cb
Pb + CHA + SA	65.9 ± 4.2 c	7.3 ± 0.6 a	30.1 ± 0.8 c	66.3 ± 3.1 b	34.7 ± 1.9 b	ND	32.7 ± a	69.3 ± 2.3 b

increased in response to D-Arg and CHA treatment while supplemental PAs mitigated the deleterious impact of PAs inhibitors. Exogenous SA also significantly decreased MDA concentrations in D-Arg and CHA-treated plants. Inhibitors of PAs synthesis decreased total GSH concentration in the shoots (Fig. 3C) but not in the roots (Fig. 3D) of Pb-treated plants. Exogenous SA increased total GSH in Pb-treated plants both in the absence or in the presence of inhibitors, especially in the roots. Shoot total ascorbate (Fig. 3E) was reduced by CHA but not by D-Arg treatment. In contrast, root total ascorbate (Fig. 3F) was significantly reduced by both inhibitors and exogenous SA did not mitigate the recorded decrease. For a given treatment, PC concentrations were always higher in the roots (Fig. 3H) than in the shoot (Fig. 3G). In both organs, D-Arg reduced PC content while CHA reduced it in the shoot but not in the roots. Exogenous SA mitigated the impact of PAs inhibitors on the shoot PC concentrations of Pb-treated plants and it strongly increased the root PC content to a similar extent whatever the inhibitor treatment. In contrast, no impacts of inhibitors were recorded on PC content in plants not exposed to Pb stress (Table S3).

Table 4
Synthesis of ethylene (in nL g^{-1}h^{-1}), concentration of putrescine (Put), spermidine (Spd), spermine (Spm) (in μmol g^{-1} FW) salicylic acid (SA; in μg g^{-1} FW), malondialdehyde concentration (MDA; in nmol.g^{-1}FW), total ascorbate (in nmol.g^{-1} FW), total glutathione (GSH; in in nmol.g^{-1} FW), total antioxidant power (estimated on 2,2-diphenyl-1-picrylhydrazyl (DPPH) reduction; in %) and phytochelatin concentration (PC; in nmol.g^{-1} FW) in roots and shoots of *Piptatherum miliaceum* cultivated during 6 weeks in the presence of 500 μM Pb, and exposed to 2-aminoethoxyvinyl glycine 2 μM (AVG; 2 μM)), methylglyoxal-bis-guanyl hydrazine (MGBG; 1 mM), exogenous putrescine (Put; 100 μM), exogenous spermidine (Spd; 100 μM) or different combinations of inhibitors and exogenous polyamines. Each value is the mean of 4 biological replicates (3 pooled plants per replicate). Standard errors are not indicated for the sake of clarity. For a given organ and a given parameter, values followed by different letters are significantly different at $P < 0.05$ according to the Tukey HSD test.

Parameter	Organ	Pb AVG	Pb+	Pb + MGBG	Pb + MGBG + Spd	Pb + MGBG + Spm
Ethylene	Shoots	2.58 b	1.12 a	3.78 c	4.02 d	3.84 cd
	Roots	2.75 *b*	0.13 *a*	4.13 *c*	4.78 *d*	4.34 *c*
Put.	Shoots	117 a	121 a	259 b	228 b	237 b
	Roots	131 *a*	187 *b*	253 *c*	301 *d*	216 *bc*
Spd.	Shoots	83 bc	78 b	41 a	94 c	32 a
	Roots	107 *b*	135 *c*	31 *a*	112 *b*	43 *a*
Spm.	Shoots	57 b	62 b	12 a	15 a	75 c
	Roots	71 *b*	88 *c*	42 *a*	51 *a*	92 *c*
SA	Shoots	125 bc	117 b	51 a	60 a	135 c
	Roots	94 *a*	85 *a*	107 *a*	108 *a*	101 *a*
MDA	Shoots	23.5 b	15.8 a	29.1 c	17.3 ab	16.1 a
	Roots	28.1 *b*	18.3 *a*	35.6 *c*	35.2 *c*	20.2 *a*
AsA	Shoots	432 b	417 b	273 a	296 a	284 a
	Roots	207 *c*	218 *c*	105 *a*	153 *b*	164 *b*
GSH	Shoots	554 c	527 c	381 a	398 ab	425 b
	Roots	463 c	482 c	303 a	325 a	396 b
DPPH	Shoots	66.4 c	71.2 d	41.5 a	45.9 a	54.2 b
	Roots	70.3 *c*	77.7 *d*	48.3 *a*	59.5 *b*	60.3 *b*
PC	Shoots	95.2 c	89.1 bc	61.3 a	76.2 b	117.4 d
	Roots	294.2 *c*	233.2 *a*	322.3 *d*	258.6 *b*	245.8 *ab*

3.3. *Impact of ethylene synthesis inhibitors and elicitor in plant response to Pb toxicity*

In Table 4, ethylene synthesis, polyamines concentrations and SA concentration are presented for shoots and roots of Pb-treated plants exposed to 2 μM AVG, MGBG 1 mM or simultaneously exposed to MGBG and Spd (100 μM) or Spm (100 μM). Application of AVG decreased ethylene synthesis to a higher extent in the roots than in the shoots. It had no significant impact on shoot polyamine concentration but increased all polyamines content in the roots. AVG had no influence on endogenous SA concentration in shoots and roots of Pb-treated plants. Application of MGBG 1 mM on Pb-treated plants strongly increased ethylene synthesis and Put concentrations but significantly decreased Spd and Spm concentrations. It also drastically reduced by more than 50 % the shoot SA content but had no impact on the root SA concentration. Supplying additional Spd to MGBG-exposed plants increased Spd content but had no impact on Spm or SA concentrations. In contrast, supplying additional exogenous Spm allowed to recover SA concentration close to plants not exposed to inhibitors at the shoot level.

As illustrated in Fig. 4, exposure of Pb-treated plants to AVG improved DW in shoots and roots (Fig. 4A) compared to plants exposed to Pb in the absence of AVG but such an impact was not observed in unstressed plants (Table S3). Exposure to MGBG induced an opposite effect and strongly reduced DW in both organs. Both exogenous Spd and

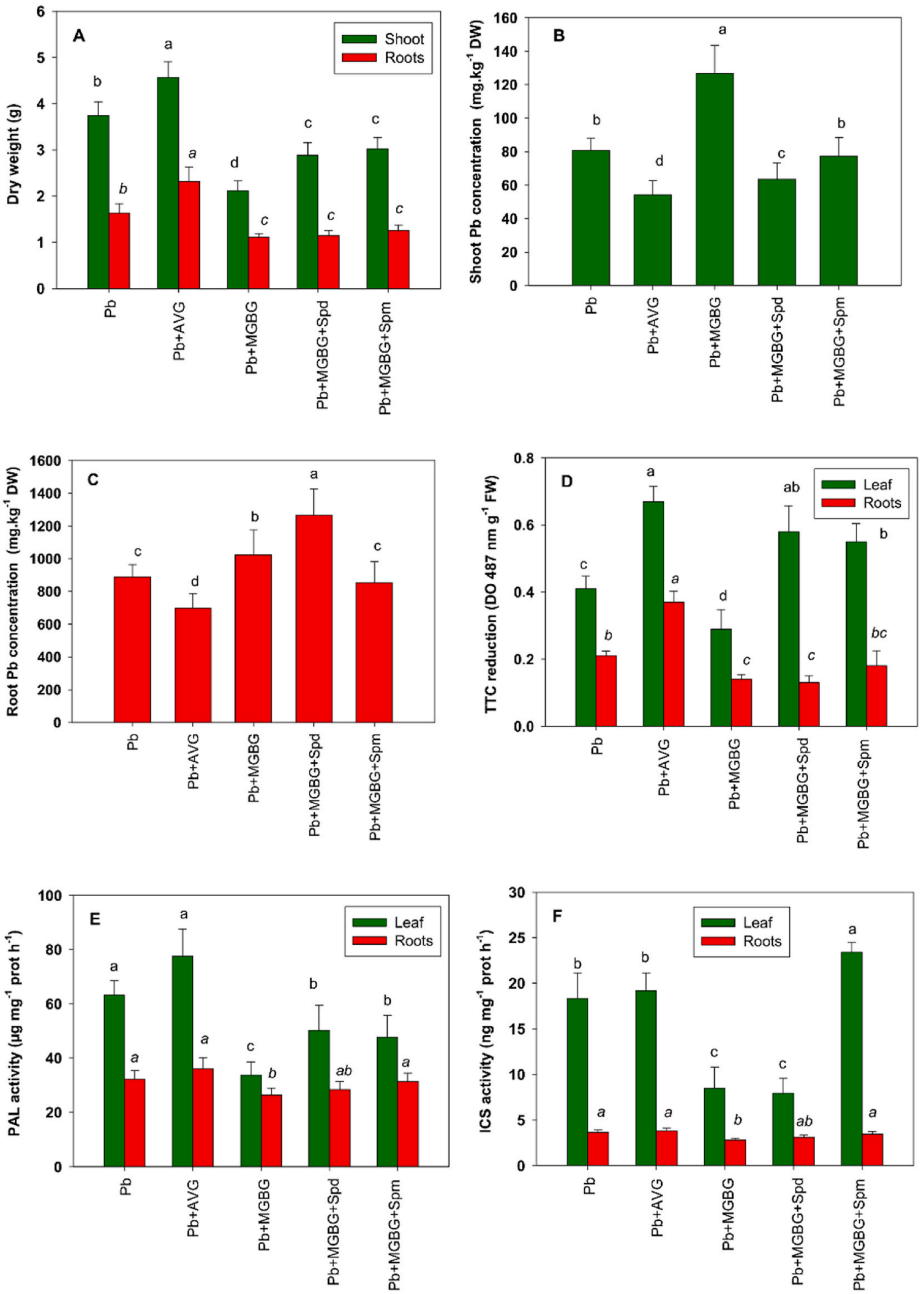

Fig. 4. Dry weights (in g; A), Pb concentrations (in mg.kg^{-1} DW; B and C), cell viability index (TTC reduction, in DO 487 nm g^{-1} FW; D) PAL (E) and ICS (F) activities in shoots and roots of *Piptatherum miliaceum* cultivated during 6 weeks in the presence of Pb 500 µM. Plants were simultaneously exposed to 2-aminoethoxyvinyl glycine (AVG; 2 µM) or methylglyoxal-bis-guanyl hydrazone (MGBG; 1 mM) in the absence or in the presence of supplemental exogenous spermidine (Spd; 100 µM) or spermine (Spm; 100 µM). For dry weight and ion content, each value is the mean of 6 replicates. For cell viability and enzyme activities, each value is the mean of 4 biological replicates (each replicate comprising three pooled plants). Vertical bars are standard errors. Values with different letters are significantly different at $P < 0.05$ according to the Tukey HSD test. Note that vertical scales are not necessarily the same for shoots and roots.

Spm allowed to partly mitigate the deleterious impact of MGBG on shoot growth but not at the root level. Plants exposed to Pb + AVG presented a lower Pb accumulation in all plant parts than plants exposed to Pb only (Fig. 4B and C). An opposite trend was recorded when MGBG was applied to Pb-treated plants, leading to a high Pb concentrations in the roots and in the shoots. Supplying Spd to MGBG-treated plants decreased Pb concentration in the shoots but increased it in the roots. In contrast, Spm had no impact on Pb distribution in plants exposed to MGBG. Cell viability (TTC test; Fig. 4D) was increased in Pb-treated plants exposed to AVG compared to plants exposed to Pb stress in the absence of AVG. Plants exposed to Pb + MGBG exhibited the lowest cell viability. Exposure to Pb + MGBG + Spd induced an increase in cell viability at the shoot level compared to Pb + MGBG – treated plants, and a similar trend was recorded for plants receiving the Pb + MGBG + Spm treatment.

AVG had no impact on PAL activities in the shoots and roots (Fig. 4E) while MGBG reduced PAL activities in the shoots. Additional Spd and Spm allowed a partial recovery of PAL activities in MGBG-treated plants. A similarly trend was recorded in the roots but the differences were not significant in most cases. Isochorismate synthase activity (ICS) at the shoot level displayed similar values in Pb and in Pb + AVG–treated plants (Fig. 4F). The recorded activities strongly decreased in the shoots of Pb + MGBG and in Pb + MGBG + Spd–treated plants. In contrast, addition of Spm to Pb + MGBG-treated plants allowed a full recovery of ICS activities which were even higher than in the Pb-treated control. The recorded activity remained low in the roots and was unaffected by the treatment, except a slight decrease in MGBG-treated plants.

Malondialdehyde, total GSH, total ascorbate, DPPH and PC concentrations are provided in Table 4 for plants exposed to Pb in the presence of ethylene effectors (AVG, MGBG) and polyamines (Spd and Spm). AVG treatment decreased MDA concentrations in shoots and roots while MGBG increased it. Addition of Spd decreased MDA in the shoots but increased it in the roots. Spm reduced MDA concentration in all organs. Addition of AVG had no impact on shoot and root ascorbate or glutathione concentration while MGBG reduced both types of compounds. The presence of additional Spd and Spm increased ascorbate content in the roots of MGBG-exposed plants but had no impact in the leaves. The DPPH values slightly increased in response to AVG but strongly decreased in response to MGBG. It has to be noticed that exogenous Spd to MGBG-treated plants increased S concentration by 26 % in the roots and by 35 % in the shoots; indeed, S concentration in MGBG-treated plants were 2.17 g kg^{-1} and 3.32 g kg^{-1} in the roots and shoots, respectively but increased up to 2.74 g kg^{-1} in the roots and 4.19 g kg^{-1} in the shoots in response to MGBG + Spd treatment (detailed data not shown). Additional polyamines improved the root (Spd) or both root and shoot (Spm) total antioxidant capacities. At the shoot level, PC concentration was slightly reduced by AVG and strongly decreased in response to Pb + MGBG and Pb + MGBG + Spd treatments. In contrast addition of Spm led to a high PC accumulation in the shoots of Pb + MGBG-treated plants. The root PC concentration was the highest in response to Pb + MGBG treatment and the lowest in response to AVG exposure.

4. Discussion

4.1. *Piptatherum miliaceum can cope with high external dose of available Pb*

The considered area was spontaneously colonized by smilo grass and in the present work, plants obtained from harvested seeds were able to cope with high concentrations of available Pb in hydroponic cultures. All cultivated plants indeed remained alive after 6 weeks of exposure to 500 μM Pb confirming the high level of Pb resistance in *P. miliaceum* (Parraga-Aguado et al., 2015; García et al., 2004; Conesa et al., 2023; Moreno-Barriga et al., 2017). Plants accumulated up to 900 mg kg^{-1} Pb in the roots and 84 mg kg^{-1} Pb in the shoots. Such high levels of accumulation however induced growth inhibition in all plant parts, although they did not affect the tillering process.

From a conceptual point of view, growth inhibition should not necessarily be considered as a symptom of injury in stressed plants and may result from the use of metabolic energy to trigger tolerance mechanisms rather than for biomass production (Lutts et al., 2004). Moreover, in the specific case of lead, several studies demonstrated that components of the cell wall limit Pb apoplastic transport and this element may precipitate extracellularly by binding to ion exchange locations in lignin and pectin (Quinet et al., 2014; Kumar and Prasad, 2018; Sofy et al., 2020; Aslam et al., 2021). Lead accumulation in the cell wall then affects cell wall extensibility and decreases cell expansion but contributes to avoid Pb entry in the symplasm (Aslam et al., 2021). A similar cell wall sequestration has been reported to efficiently prevent Cd toxicity in safflower (Pourghasemian et al., 2019). Although Pb subcellular distribution was not analyzed in the present study, the fact that electrolyte leakage did not increase in Pb-treated roots (Table 1) partly supports this hypothesis but the recorded decease in cell viability and increase in MDA concentrations (Table 1) in both shoots and roots indicated that growth inhibition in our plants also occurred as a result of Pb toxicity.

4.2. *Polyamines and salicylic acid contribute to lead tolerance in Piptatherum miliaceum*

Polyamines (Quinet et al., 2014; Rady et al., 2016; Tang et al., 2017; Taie et al., 2019; Spormann et al., 2021; Gu et al., 2022) and salicylic acid (Freeman et al., 2005; Zaid et al., 2019; Sharma et al., 2020; Sofy et al., 2020) are frequently reported to be involved in plant response to heavy metal toxicity. In the present study, concentrations of all PAs increased in *P. miliaceum* exposed to lead 500 μM. Any decrease in PAs titer through the use of specific inhibitors had a deleterious impact on plant response exposed to Pb and in most cases complementation with exogenous polyamines allowed to mitigate or even completely repressed the deleterious impact of inhibitors.

D-arginine is an inhibitor of ADC (EC 4.1.1.19) and reduced Put synthesis without suppressing it since the alternative pathway through ODC (EC 4.1.1.17) is still operating (Fig. 1A). A reduced Put availability for subsequent synthesis of Spd and Spm explain that all PAs were reduced in response to this treatment (Table 3; Table S3). In D-Arg-treated plants, growth was inhibited while Pb content increased in the shoots but decreased in the roots (Fig. 3C and D). Cell viability was deeply affected and electrolyte leakage as well as MDA strongly increased. Reducing PAs concentration thus had a strong detrimental impact on Pb-treated plants. It is well known that PAs control numerous functions in plants and regulate cell division processes and cell elongation (Rady et al., 2016; Malik et al., 2022; Tyagi et al., 2023) so that a decrease in PAs concentration may explain the recorded growth inhibition. An expected negative correlation was found between Pb content and dry weights for shoots ($r = -0.92$; $P < 0.01$) and roots ($r = -0.88$; $P < 0.01$). Correlation analysis (Table S4) revealed a positive correlation between cell viability index on the one hand and Spd concentration ($r = 0.94$, $P < 0.01$ for shoots; $r = 0.89$, $P < 0.01$ for roots) and Spm concentration ($r = 0.96$, $P < 0.01$ for shoots, $r = 0.94$, $P < 0.01$ for roots) on the other hand.

Because of their cationic features at cytosolic pH, PAs interact with negatively charged biomolecules such as DNA and protein and stabilize cell membranes (Legocka et al., 2015; Roy et al., 2024). A drop in endogenous PAs may thus explain an increase in electrolyte leakage. Polyamines also assume crucial antioxidant functions. They modulate the level of endogenous antioxidant (Malik et al., 2022; Taie et al., 2019; Tyagi et al., 2023) and the present study demonstrates that D-Arg decreased GSH concentrations in the shoots and AsA in the roots (Fig. 4). Polyamines also stimulate enzymatic antioxidants such as SOD, GPX, GR (Tang et al., 2017; Gu et al., 2022) and PAs themselves were reported to act as free radical scavengers and eliminate ROS such as $^1O_2$ or OH· (Spormann et al., 2021). Supplying Put to plants cultivated in the presence of D-Arg allowed to recover a normal polyamine profile (Table 3) and simultaneously suppressed the deleterious symptoms induced by D-Arg on Pb-treated plants, confirming their crucial role in the management of Pb toxicity in *P. miliaceum*.

Cyclohexylammonium (CHA) is an inhibitor of Spd synthase (EC 2.5.1.16; Fig. 1A) and in contrast to D-Arg, it induced a decrease in the triamine Spd and the tetramine Spm but not in the diamine Put (Table 3). It had similar impact than D-Arg on dry weight, Pb distribution, electrolyte leakage, MDA, and was even more deleterious than D-

Arg on cell viability and DPPH; this prompted us to consider that Spd and/or Spm but not Put are involved in the management of Pb toxicity in *P. miliaceum*. In fact, Put synthesis should be tightly regulated in plants and it may become toxic beyond a certain level and may be responsible for apoptotic cell death at high concentrations (Takao et al., 2006; Qiao et al., 2014). In *P. miliaceum* exposed to Pb stress, plants treated with CHA + Put exhibited a very high Put concentration (Table 2) and the toxicity of this treatment is exemplified by the fact that it aggravated Pb toxicity symptoms in terms of weight, cell viability and electrolyte leakage (Fig. 2) and induced high MDA concentration (Fig. 3) in relation to a decrease in antioxidant phenolics and DPPH capacity (Table 3).

As an inhibitor of S-adenosylmethionine decarboxylase, MGBG drastically reduced Spd and Spm concentration (Fig. 1A). Providing Spd to CHA-treated plants reduced Pb in the shoots and increased it in the roots. Since exogenously provided Spd may still be converted to Spm in CHA-treated plants (Fig. 1A), this does not allow us to discriminate between the impacts of the two polyamines on Pb distribution (Fig. 1A). However, such a conversion was not anymore possible in MGBG-treated plants because of the lack of dSAM for subsequent synthesis of Spm from Spd (Fig. 1A). In those plants, providing Spd allowed to increase Pb concentration in the roots and decreased it in the shoots (Fig. 4) while providing Spm had no similar effect, leading us to consider that Spd but not Spm may reduce Pb translocation to the aerial parts. Tang et al. (2017) and Gu et al. (2022) reported a similar situation in *Salix matsudana* and rice, respectively, and considered that Spd-induced root accumulation of Pb protected the aerial part from injuries to the photosynthetic machinery.

In the present study, spermidine was also shown to improve S nutrition in Pb-stressed plants and this might be interpreted as an attempt to increase GSH synthesis for subsequent polymerization in PC. However, our data show that Spd had only a minor impact on leaf and root PC concentration. In contrast, exogenous SA strongly increased GSH and PC concentrations, especially at the root level (Fig. 3H; correlation were highly significant between SA and GSH ($r = 0.91$, $P < 0.01$) as well as between SA and PC ($r = 0.94$, $P < 0.01$) (Table S4). Such an increase occurred both in the absence of PAs inhibitors and in the presence of D-Arg and CHA when the endogenous concentrations of PAs were low (Table 2). This suggests that SA directly stimulated GSH and PC synthesis independently of PAs. Nazar et al. (2015) reported that SA increased ATP-sulfurylase and serine acetyl transferase in *Brassica juncea* leading to an increase in cysteine and GSH concentrations. According to Freeman et al. (2005), this process mediated by SA is directly involved in nickel tolerance of Ni-hyperaccumulators from the *Thlaspi* (syn. *Nocaea*) genus. Hence, a high PC content induced by SA in Pb-treated plants of *P. miliaceum* may help the roots to cope with internal Pb concentrations 10 times higher than those recorded in shoots.

Lead exposure induced an increase in SA content in shoots and roots (Table 1). Inhibitors of PAs synthesis such as D-Arg and CHA also reduced endogenous SA which could be restored by exogenous PAs (Table 2) confirming that the synthesis of the two classes of compounds display complex interactions (see below). Exogenous SA allowed to partly reduce the deleterious impact of inhibitors in the absence of additional exogenous PAs on the shoot dry weight (Fig. 2A), to mitigate oxidative stress as indicated by a lower MDA content (Fig. 3A and B) and to reduce electrolyte leakage (Fig. 2G and H). These impacts of exogenous SA were observed for plants exposed to inhibitors of PAs synthesis but not for plants exposed to Pb alone and this suggests that in this latter case, endogenous titters of SA were sufficient to help the stressed plants to cope with Pb toxicity and that supplemental SA did not afford any additional advantages for the recorded parameters.

In Syrian beancaper (*Zygophyllum fabago*, another plant species spontaneously colonizing the contaminated area of La Union), López-Orenes et al. (2014) demonstrated that SA pretreatment reduced Pb accumulation in both roots and aerial parts. No similar effect was detected for Pb in *P. miliaceum* since Pb accumulation and distribution remained unaffected by SA (Fig. 2C and D). Hence, SA-induced increase in cell viability resulted from an improvement of cell tolerance and not from stress avoidance linked to endogenous Pb decrease. López-Orenes et al. (2020) provided evidence in *Z. fabago* that SA was involved in the oxidation of coniferyl alcohol and ferulic acid by Pb and appeared to regulate cell wall stiffening and its capacity to sequester metal ions. Several studies dealing with the positive impact of SA on heavy metals stressed plants considered oxidative stress-related parameters (Zaid et al., 2019; Sharma et al., 2020; Sofy et al., 2020; Liu et al., 2022; Zheng et al., 2022; Li et al., 2023). Exogenous SA increased GSH concentrations but had no impact on AsA (Fig. 3E and F). For a given pool of antioxidant, activities of enzymes involved in the Asada cycle (ascorbate peroxidase, glutathione reductase, monodehydroascorbate reductase) may be activated and could reduce oxidative stress (Kunert and Foyer, 2023). Salicylic acid increased phenolic compounds in shoots of CHA-treated pants and in roots of D-Arg and CHA-treated ones leading to an increase in DPPH (Table 4). Moreover, SA beneficial impact on stressed plants is not specific to Pb toxicity and this compound assume positive functions in drought- and salt-treated plants (Askari and Ehsanzadeh, 2015; Gharbi et al., 2016; Yousefzadeh-Najafabadi and Ehsanzadeh, 2021).

4.3. *Ethylene appeared to be involved in lead toxicity*

As far as *P. miliaceum* exposed to Pb toxicity is concerned, ethylene obviously appeared to have a negative impact on plant response to Pb toxicity. Indeed, reducing ethylene synthesis through AVG (inhibitor of ACC oxidase; Fig. 1A) improved the plant behavior for all recorded parameters since it increased dry weight (Fig. 4A) and cell viability (Fig. 4D) but also reduced MDA concentration and increased DPPH (Table 4) even if it had no impact on ascorbate and GSH content. At the opposite, MGBG strongly increased ethylene synthesis by inhibiting S-adenosylmethionine decarboxylase involved in PAs synthesis (Fig. 1A). MGBG was by far the most detrimental treatment for Pb-exposed plants in *P. miliaceum*. Negative correlation was found between ethylene synthesis and cell viability for shoots ($r = -0.94$, $P < 0.01$) and for roots ($r = -0.88$, $P < 0.01$).

It is noteworthy that AVG reduced Pb concentrations in all parts of the plant while MGBG increased it (Fig. 4) suggesting that ethylene contributes to Pb absorption in smilo grass. Although heavy metals-induced oversynthesis of ethylene is well established, the impacts of ethylene on ions transporters remain poorly documented. This is especially the case for Pb which is a non-essential element and manly enters root symplasm through non-specific Ca^{2+}-channels (Abedi et al., 2022). Some transporters such as PFR12, ATM1 or the leucine-rich repeat (LRR) protein contribute to Pb resistance by regulating Pb efflux (Cao et al., 2009; Kumar and Prasad, 2018). An inhibiting effect of ethylene on this efflux process could not be ruled out. However, Cao et al. (2009) demonstrated that in *Arabidopsis thaliana*, the *EIN2* gene which is a central component of ethylene signaling is involved in the expression of *AtPDR* coding for a pleiotropic drug resistance ABC transporter involved in Pb efflux. This is in contradiction with our hypothesis on *P. miliaceum* but there is no exhaustive evidence that smilo grass which is well adapted to harsh environments and polluted substrates (Zavas et al., 1996; García et al., 2004; Parraga-Aguado et al., 2015; Conesa et al., 2023), should behave like the model plant species *A. thaliana*. Moreover, Zuo et al. (2023) recently identified an Agamous-like MADS box gene (*EcAGL*) from *Erigeron canadensis* which modulate the expression of ACC synthase gene involved in ethylene synthesis and which also influence the expression of genes coding for heavy metal transporters from HMA and NRAMPs families. It has to be mentioned that AVG is not fully specific to ACC oxidase since it inhibits a range of enzymes that depend on pyridoxal-5′-phosphate (PLP) as a cofactor (Le Deunff et al., 2019) and additional impact of this inhibitor on plant metabolism cannot be ruled out.

In contrast to the AVG treatment, MGBG strongly reduced Spd and Spm synthesis and it is not clear if its deleterious effect resulted only from ethylene oversynthesis or from a lack of Spd and Spm. Supplying Spd and Spm to MGBG-treated plants allowed to increase root DPPH but only Spm was able to increase root GSH and to decrease root MDA comparatively to MGBG-treated plants (Table 4). However, supplying Spd on MGBG-treated plants reduced Pb content in the shoots but strongly increased it in the roots (Fig. 4B and C), strengthening our hypothesis on the role of Spd in decreasing root to shoot Pb translocation in *P. miliaceum*.

4.4. Spermine contributes to salicylic acid synthesis in Pb-treated plants through an increase in isochorismate synthase activity

Several studies focused on the impact of SA and PAs metabolism. According to Pál et al. (2021), a wide range of abiotic stresses including drought, salt, chilling, UV-B exposure and heath induce SA accumulation which directly influence PAs biosynthesis. Canales et al. (2019) demonstrated that SA regulates PAs biosynthesis through an increase in Spm and a decrease in Put resulting from downregulation of ADC gene and upregulation of the SADMC gene in oat exposed to water stress. Similarly, exogenous application of 10 μM SA increased PAs production in the halophyte species *Solanum chilense* by activating *ADC1* and *ADC2* gene expression in the shoot (Gharbi et al., 2016). In *Arabidopsis thaliana* exogenous application of SA also induced Put accumulation involved in plant response to infection by *Pseudomonas syringeae* (Rossi et al., 2021). The impact of SA on PAs metabolism may be influenced by environmental conditions such as light and ethylene could modulate this influence (Takács et al., 2021). Surprisingly, the reverse impact of PAs on SA biosynthesis receive quite less attention. Our work provides for the first time to the best of our knowledge circumstancial evidences that PAs are involved in regulation of SA biosynthesis in *P. miliaceum* exposed to Pb toxicity but that the modalities of this regulation are not the same in shoots and roots.

At the shoot level, D-Arg and CHA drastically reduced PAs content but also decreased SA concentration (Table 2) and a similar effect was recorded for MGBG-treated plants (Table 4). Providing Put to D-Arg treated plants and Spd to CHA-treated ones not only allowed to restore PAs profile but also restored shoot SA concentration. In contrast, applying Put to CHA-treated plants or Spd to MGBG-exposed ones did not restore the shoot SA content. This leads us to hypothesize that Spm may trigger SA accumulation in shoots of Pb-treated plants of *P. miliaceum* and this hypothesis is further supported by the fact that applying Spm in the presence of MGBG fully restored a normal shoot SA content (Table 4). When data from all treatments were pooled together, we found a highly positive correlation ($r = 0.91$; $P < 0.01$; Table S4) between shoot Spm and shoot SA. A completely different trend was recorded at the root level: in this organ, SA concentration was never significantly affected neither by PAs biosynthesis inhibitors nor by exogenous PAs treatment (Tables 2 and 4).

Salicylic acid in plants is synthesized by two alternative pathways (Fig. 1B). The phenylalanine ammonia lyase pathway takes place in the cytosol and it implies the conversion of phenylalanine to *trans*-cinamic acid which is then converted to benzoic acid by the abnormal inflorescence meristem 1 (AIM1) protein. The last step converting benzoic acid to SA is presumably catalyzed by benzoic acid hydroxylase which has not yet been purified (Lefevere et al., 2020). The second pathway takes place within chloroplasts and it implies the conversion of chorismate to isochorismate by ICS, and the synthesis of SA from isochorismate by isochorismate pyruvate lyase (IPL) (Chen et al., 2009; Nugroho et al., 2001). The relative importance of the two pathways differs between plant species and also depends on environmental conditions. In *P. miliaceum* exposed to Pb toxicity, PAL activity was recorded in shoots and roots while ICS activity was mainly detected in the shoots but not in the roots (Table 1). This could be explained by the chloroplastic localization of ICS. In the shoots of *P. miliaceum* exposed to Pb toxicity, any decrease in Spm concentration implies a concomitant decrease in ICS activity (Table 4). In contrast providing Spm to MGBG-treated plants strongly increased the shoot ICS activity while Spd did not (Fig. 4F). These observations lead us postulate that Spm may regulate ICS activity in the shoots which in turn would increase SA synthesis in photosynthetic tissues. At the root level, ICS was almost not present due to the lack of mature chloroplasts and very low activities in proplastids, and SA synthesis is regulated by PAL activity only. Since root PAL activity remained unaffected by PAs inhibitors and exogenous PAs (Table 3), this could explain why SA concentrations remained constant in non-photosynthetic root tissues.

5. Conclusions

Piptatherum miliaceum can cope for long periods with high external Pb concentration since it remained alive after 6 weeks of exposure to 500 μM Pb and it accumulated up to 84 mg kg^{-1} DW and 900 mg kg^{-1} DW in the shoots and roots, respectively. Despite the recorded growth inhibition, this species is a promising material for phytomanagement of Pb-contaminated sites. Polyamines and salicylic acid assume positive roles in plant defense against Pb toxicity but these compounds interact differently in shoot and roots. Polyamine contribute to cell viability and maintenance of membrane integrity. They reduce oxidative stress and increase the antioxidant power of stressed plants. Spermidine contributes to reduce Pb root-to-shoot translocation and improves S absorption for subsequent synthesis of glutathione. Salicylic acid strongly increases synthesis of glutathione and protecting phytochelatins at the root level. Spermine regulates salicylic acid synthesis through an increase in isochorimate synthase activity (ICS; EC 5.4.4.2) in the aerial part of the plant only, while phenylalanine ammonia lyase (PAL; EC 4.3.1.5) is the only enzyme involved in salicylic acid synthesis at the root level. Additional experiments, such as qPCR analysis would help in the future to determine whether spermine indeed regulates SA levels through modulation of ICS gene expression. Salicylic acid improves cell viability in Pb-stressed plants through an increase in glutathione synthesis. Ethylene has a negative impact on Pb resistance and increases Pb absorption. High concentrations of polyamines and salicylic acid may be regarded as suitable criteria for identification of Pb-resistant populations in *Piptatherum miliaceum*.

CRediT authorship contribution statement

Stanley Lutts: Writing – original draft, Supervision, Funding acquisition, Conceptualization. **Beatrice Falcinelli:** Investigation, Formal analysis. **Nolan Regnier:** Investigation, Formal analysis, Conceptualization. **Laurence Monin:** Investigation. **Nicolas Goffin:** Investigation. **Monika Patel:** Writing – review & editing, Conceptualization. **Paolo Benincasa:** Writing – review & editing, Supervision, Funding acquisition.

Ethic approval

The present study contains no investigation with human participants or animals performed by any authors.

Availability of data and materials

The data supporting this study’s finding are available from the corresponding author upon reasonable request.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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Data availability

Data will be made available on request.

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