Influence of Aluminum at Low pH on the Rhizosphere Processes of Masson Pine (Pinus Massoniana Lamb)

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Research Article

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Influence of aluminum at low pH on the rhizosphere processes of Masson Pine (*Pinus massoniana* Lamb)

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Abstract

Trees in general are very tolerant of aluminum (Al, mainly Al^{3+} at pH ≦ 5.0), and the small effects seen in the contaminated soils may mislead people that the contamination is unimportant. We believe that the assessments with Al-sensitive Masson pine could have revealed a bigger difference. The key point of this study was to characterize the Al toxicity for Masson Pine. The objectives were to discover the specific eco-physiological relationship between pine roots and rhizosphere Al, and to investigate the Al effects on several parameters, measured in the rhizosphere of Masson pine. Masson pine seedlings were cultivated on a hydroponic setup. Through comprehensive laboratory dose-gradient experiments, Al-triggered composition of the root-released compounds and several rhizospheric parameters were determined by chromatography or spectroscopy. This study gives an important evidence of the Al-toxicity effects on the composition of root-released compounds and the root growth of Masson pine. Results showed that higher rhizospheric Al at pH 4.5 might contribute to increased release of sugars, and also could stimulate the release of oxalic acid and malic acid. The total of secreted amino acids were correlated with the rhizosphere Al. Zero additional Al induced no rhizosphere pH elevation, but Al-induced rhizosphere acidification (pH from 4.50 to 4.22) was observed at Al 100 μM. Greater additions of Al (>300 μM) suppressed the rhizosphere acidification at pH 3.92. Added Al had a negative effect on the dry weight of pine roots, but an opposite effect on Al accumulated in the roots was observed. The four endogenous hormones were also determined in the pine roots. Gibberellic acid (GA_3) decreased, whereas abscisic acid (ABA) increased simultaneously with the addition of Al. Their inflexional concentrations were most frequently observed at 100 μM, which might be the threshold of Al toxicity for

Masson pine. The secondary metabolites assayed have been studied in relation to the rhizospheric Al. The rhizosphere Al species at low pH can trigger pine roots to release the sugars (glucose, fructose + aldose), organic acids (oxalic acid, and malic acid), amino acids, secondary metabolites, and endogenous hormones during their growth. Meanwhile it also affected the growth of pine roots. This is an extensive study, which can help understanding the toxicity of Al to this important pioneer species of acid forest soils in south China.

Keywords Aluminum toxicity · Low pH · Rhizosphere · Root-released compounds · Masson pine (*Pinus massoniana* L.)

Introduction

Masson pine (*Pinus massoniana* Lamb) is a widely distributed native pioneer species, which is grown on the acid forest soils in south China. This tree grows rapidly and is economically important. It shows an inheritable tolerance to environmental stresses, including acidic aluminum (Al) stress (Wu et al. 2009). Masson pine has shown symptoms of die-back under the influence of atmospheric acid deposition. Acid deposition has led to extensive soil acidification that comprise up to 50% of the world's potentially arable lands. Phytotoxic Al ion (mainly Al^{3+}) may threaten the integrity of forest ecosystems as a result of acid deposition. Acidification may lead to forest soil degradation, affecting the soil functions (Li et al. 2014). The Al released into the soil solution is usually well below 50 μM at pH > 5.5, but rises 100-fold at pH 4.5, which is of risk for the growth of sensitive plant species (Wang et al. 2006; Yang et al. 2015). High concentrations of Al may disrupt plant root functions and the metabolic changes

53 associated with root-released compounds. The root system, especially root apex, is the
54 critical site for Al toxicity. Kidd et al. (2001) and Tolrà et al. (2005) wrote about maize
55 and *Rumex acetosa* L., respectively. Nowak and Friend (2005) also observed Al
56 resistance in the root tips of slash pine. Normally, plant roots excrete low molecular
57 weight organic molecules into plant rhizosphere to adapt various stressful circumstances.
58 Study of the root-released compounds holds great promise for revealing the effects of Al
59 on tree rhizosphere.

60 Rhizosphere is an important root-soil interface for releasing organic compounds,
61 intense nutrients exchange and microbial activity, and also a gateway for potentially toxic
62 pollutants such as Al, in which normal root physiological action is greatly influenced by
63 root-released compounds (Hinsinger et al. 2005). Currently, the association between Al
64 chemistry of tree rhizosphere and soil acidification is an important concern (Rehmus et al.
65 2014; Hirano et al. 2012). Osawa and Matsumoto (2002) and Nguyen et al. (2003)
66 observed the effects of acid Al on rhizosphere ecological action. Several investigators
67 reported that the efflux of low molecular weight organic anions from root apices could
68 protect the root by chelating and thus detoxifying Al in the rhizosphere (Ma and
69 Furukawa 2003). Barceló and Poschenrieder (2002) reviewed immobilization of the cell
70 wall, selective permeability of the plasma membrane, formation of rhizosphere pH barrier
71 and evolution of Al-tolerant enzymes in higher plants under Al stress.

72 Numerous Al toxicity mechanisms have been proposed (Imadi et al. 2016). They can
73 be grouped into two categories: exclusion of Al from the roots, and detoxification of Al
74 ions in the plants (Poschenrieder et al.2008). Putative exclusion mechanisms proposed
75 include binding of Al in the cell wall, an Al-induced rhizosphere pH barrier, and

root-released Al-chelating compounds (Ma and Furukawa 2003; Liang et al. 2013). Organic acids have been suggested to play a role both in Al exclusion, via release from the root, and Al detoxification in the apoplasm or rhizosphere, where low molecular weight organic acids could chelate Al and reduce or prevent its toxic effects at the cellular level (Zhang et al. 2014; Yang et al. 2017).

Trees in general are very tolerant of Al, and the small effects seen in the contaminated soils may mislead people that the contamination is unimportant. Our study found that the assessments with Al-sensitive Masson pine would have revealed a bigger difference. The purposes of this study were to assess the effects of 17 Al levels at low pH in hydroponic culture of Masson pine on the root growth, Al uptake and accumulation. The 17 dose-gradients of Al concentrations were selected from zero to 750 μM, based on our earlier study (Wang et al. 2006; Wang et al. 2015b). Also, we measured the Al-induced changes in the root-released compounds of sugars, organic acids, amino acids, secondary metabolites, as well as rhizosphere pH and the endogenous hormones in the roots.

Materials and methods

Seedling growth and treatment

Seeds of Masson pine (China Zhejiang Forestry Science Institute) were surface-sterilized in a 75% ethanol solution for 10 min, rinsed in running tap water for 20 min, and washed with deionized water (Millipore, Eschborn, Germany) three times. Seeds were soaked in deionized water for 24 h, germinated for 48 h, and then cultivated under a hydroponic culture system.

A 350 L nutrient solution containing the following concentrations of mineral was prepared (in μM): 250 NH_4NO_3, 60 KH_2PO_4, 220 K_2SO_4, 188 $CaCl_2$, 62 $MgSO_4$ and 95 Fe-EDTA, 46 H_3BO_3, 0.3 $CuSO_4$, 0.1 $(NH_4)_6Mo_7O_{24}$, 9.2 $MnSO_4$, 0.8 $ZnSO_4$ (van Schöll et al. 2005). The mixed solution was divided into 17 equal parts. Different amounts of $AlCl_3$ were added to each part to reach concentrations of 0, 7.5, 15, 30, 45, 60, 75, 100, 150, 200, 250, 300, 350, 400, 450, 600 and 750 μM. Then the pH values of these solutions were initially adjusted to 4.5 using HCl or NaOH and checked and adjusted again three times a week. The nutrient solution without Al was used for control plants. The solutions were aerated by pumps, which connected the containers with pump lines. The nutrient solutions were changed regularly every 10 days. The seedlings from each treatment were harvested after 100 d of incubation for Al determination. To have Masson pine seedlings in a hydroponic and non-sterile system for 100 days, it is likely that contaminants such as fungi or bacteria can grow as well and establish on the root surface. Ultraviolet radiation was used to disinfect the growth chamber and the nutrient solutions prior to use.

The seedlings were transplanted into a 25-L pot containing nutrient solutions (25 seedlings per pot) and incubated in a LRH-250-G growth chamber at 25 ± 2 °C. When the seedlings reached 8 cm, they began to cultivate with the nutrient solutions containing different Al^{3+} concentrations. About 30 mL of fresh nutrient solution was added every 12 h. The seedlings were grown in a controlled culture box with a 12 h light/12 h dark cycle under 40 W m^{-2} light. The light/dark temperatures were set at 25/20 °C, and relative humidity was kept at 65%. Concentrations of Al in the solution were measured and replenished every 12 h.

120 The root-released compounds were collected following the procedures described in
121 our previous studies (Wang et al. 2006; Wang et al. 2015b). Briefly, the roots were
122 treated with deionized water, and then exposed to a 1 L 0.5 mM $CaCl_2$ solution (pH 4.5)
123 with corresponding Al level for 24 h and then washed with 100 mL of deionized water
124 (25 seedlings per measurement). To avoid interaction between Al and other nutrients such
125 as P, a simple salt solution containing 0.5 mM $CaCl_2$ was used as the basal treatment. The
126 above-mentioned Al treatment solution was placed on a shaker, centrifugally separated
127 (60 rpm) for 2 h, and filtered. The filtrate was divided into two equal parts. One part was
128 used to measure pH, and the other part was concentrated to 50 mL under vacuum and
129 analyzed for sugars, amino acids, organic acids and secondary metabolites. The solution
130 pretreatment was performed at 4 °C.

131 **Analysis**

132 Rhizosphere pH

133 The pH electrode was placed in the filtrate, and pH value was read as soon as the PHS-25
134 pH-meter (Shanghai Precision Instruments Co., China) stabilized.

135 Sugar

136 A 5 mL filtrate was hydrolyzed with 10 mL of 4% H_2SO_4 under vacuum at 110 °C for 1 h.
137 After cooling, the hydrolysate was washed with deionized water, filtered (Whatman No.2,
138 USA) and dried at 60 °C (also under vacuum) by a rotary evaporator. The dried sample
139 was then dissolved in 5 mM H_2SO_4. The sugar (monosaccharide) in the hydrolysate was
140 separated and quantified by injecting 10 μL into a HPLC (Waters 600, USA) with RI × 4
141 detector, equipped with a Sugar-pak^{TM} 1. P/N 85188 column (Waters, USA). The column

142 temperature was 85 °C. Milli-Q water was used as the mobile phase with a flow rate of
143 0.6 mL min^{-1}.

144 Organic acids

145 The filtrate was passed through a cation exchange column (16×14 mm) filled with 5 g of
146 Amberlite IR-120B resin (H^+ form, Shanghai Chemical Reagent Co., China), followed by
147 an anion-exchange column (16×14 mm) filled with 2 g of Dowex 1×8 resin (100–200
148 mesh, format form; Shanghai Chemical Reagent Co., China). The organic acids retained
149 on anion-exchange resin were eluted by 1 M HCl, and the eluate was concentrated. 10 μL
150 concentrating solution was injected onto an Aminex HPX-87H column (7.8 mm i.d. ×
151 300 mm, 9 μm). The quantitative determination of organic acids was carried out with
152 electrospray ionization-tandem mass spectrometry (ASE-SPE-LC-ESI-MS/MS). The
153 mobile phase used was 5 mM H_2SO_4 at a flow-rate of 0.5 mL min^{-1}. Detection was at a
154 wavelength of 210 nm. Column temperature was 50 °C (Wang et al. 2015a).

155 Amino acids

156 A 5 mL filtrate was hydrolyzed with 8 mL of 6 M HCl under vacuum at 110 °C for 24 h.
157 After cooling, the hydrolysate was washed with deionized water, filtered (Whatman No.2,
158 USA) and dried at 60 °C (under vacuum) by a rotary evaporator. The dried sample was
159 then dissolved in 0.01 M HCl. The amino acids in the hydrolysate were separated and
160 quantified by injecting 50 μL into a Hitachi 835-50 Amino Acid Automatic Analyzer
161 (Hitachi, Japan) equipped with a 2.6 mm × 150 mm ion exchange column coated with
162 resin 2619#. The column temperature was 53 °C. Sodium citrate buffers (pH 3.3, 4.3, and
163 6.3) were used as eluents with a flow rate of 0.225 mL min^{-1}. The light absorbance of the
164 amino acids was detected with a 166 Detector (Beckman Instruments) at 570 nm.

165 Secondary metabolites

166 Secondary metabolites were analyzed using a Finnigan Trace DSQ GC-MS (USA) in
167 selected ion mode (SIM). The capillary column used was a DB-5MS (30 m × 0.25 mm id
168 × 0.25 μm film thickness). The carrier gas was helium. A split/splitless injector in the
169 splitless mode was used. The inject volume was 1.0 μL (Tikhomiroff and Jolicoeur
170 2002).

171 Aluminum

172 The fresh Masson pine roots were treated with deionized water (25 seedlings per
173 treatment) and cut into small pieces, which were dried at 70 °C for 48 h to determine their
174 dry weight (van Schöll et al. 2004). The dried roots were weighed, ground, acid-digested,
175 filtered, and finally concentrated to a certain volume. The total Al was determined by
176 inductively coupled plasma atomic emission spectrometer (ICP-AES, PS-1000AT, USA)
177 (Wang et al. 2012).

178 Endogenous hormones

179 Endogenous hormones were analyzed using a LC-ESI-MS/MS system. Ten μL of the
180 above-mentioned solution for Al determination was injected onto a KC-811 column.
181 Detection was at a wavelength of 254 nm. The mobile phase used was a mixed solution
182 (methanol/water/acetic acid 50:49.3:0.7, V/V/V) at a flow rate of 0.6 mL min^{-1}. Column
183 temperature was 35 °C. Quantification was based on the LC-ESI-MS/MS peak areas
184 found for the base peaks of single hormones (Wang et al. 2016).

Statistical analysis

The data presented in Figures 1–4 were the mean and standard deviation (SD) of nine replicated treatments (nine measurement, one measurement for one pot), which was an expensive long-term experiment (from 2006 to 2018). Recovery of the extraction/concentration procedure was evaluated. For each variable, the normality of the distribution was tested with a Shapiro–Wilk test. Levels of significance were $P < 0.0001$. The wide range of Al concentration treatments tested was studied to analyze the dose-response relationship. The curves trends, linear or quadratic, attached to the response variables were observed in Figures 1–4.

Results

It was found that roots released organic acids; however, it was only known to a lesser extent, that roots also released other compounds such as sugars, amino acids, or phenolic compounds. Some of these organic molecules can bind Al and, thus, potentially detoxify the phytotoxic Al ions. The detailed description for the experimental results was given in the following sections.

Al-triggered variation of root-released compounds

Rhizosphere pH

The variation of Al-contaminated rhizosphere pH was assessed in the presence and absence of Al (Fig. 1a). At lower Al level (100 μM), the addition of Al caused the rhizosphere pH to decrease from 4.50 to 4.22. However, when Al level varied from 100 to

300 μM, the rhizosphere pH decreased rapidly from 4.22 to 3.92. At higher Al level (≧ 300 μM), there was no clear trend for a further decrease in the rhizosphere pH values.

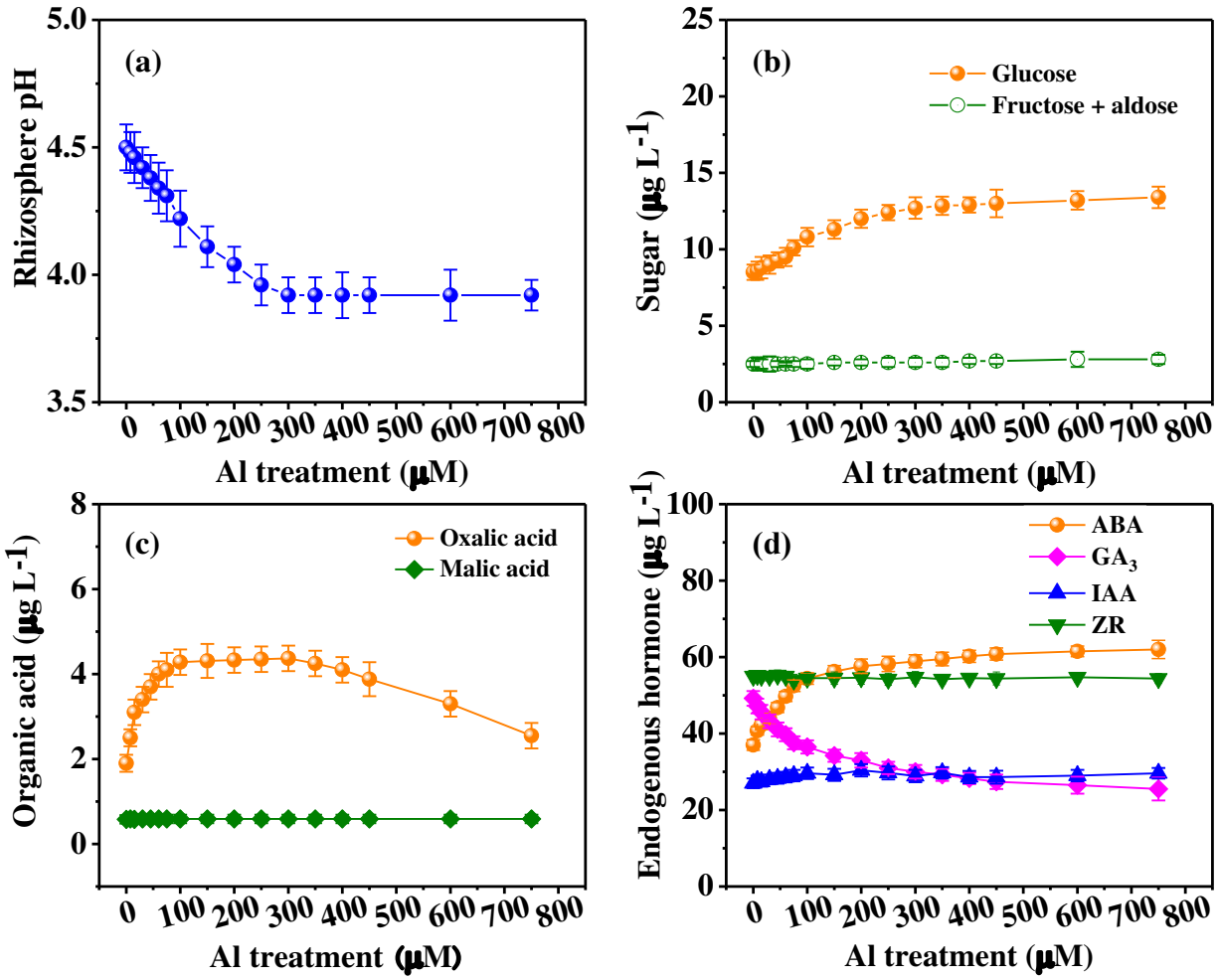

Fig. 1 Al-stimulated variation of the rhizosphere pH (a), sugars (glucose, fructose + aldose) (b), organic acids (oxalic and malic acids) (c), and endogenous hormones (d) released from Masson pine roots. All data present here are expressed as arithmetic means of nine observations ± SD (standard deviation). Error bars represent SD from n = 9 replicates.

Sugars

Glucose, fructose and aldose were identified in the root-released compounds. Only glucose was influenced by rhizosphere Al levels. Its concentrations increased proportionally with Al levels. When Al concentrations were varied from 0 to 100 μM, the

218 increase in glucose was below 15 %, which was significantly different from the other
219 higher Al levels indicated in Fig. 1b. However, the released amounts of fructose and
220 aldose were very low in the given Al treatment range. No differences were found in the
221 release rates of fructose and aldose or the pattern of response to increasing Al
222 concentrations.

223 Organic acids

224 Oxalic and malic acids were determined using ASE-SPE-LC-ESI-MS/MS system (Wang
225 et al. 2015a). The occurrence of Al-induced oxalic acid in the Masson pine rhizosphere
226 and its special relevance concerning Al levels were presented in Fig. 1c. Increasing
227 rhizosphere Al from 0 to 100 μM slightly reduced the release of oxalic acid. Interestingly,
228 high Al^{3+} exposure (>300 μM) triggered a significant small stimulation in oxalic acid. In
229 contrast, there was no clear varying trend for the root release of malic acid.

230 Amino acids

231 The effects of rhizosphere Al on the root-released amino acids were presented in Fig. 2.
232 In the blank assay, twelve amino acids, including alanine (Ala), asparagine (Asp), cystine
233 (Cys), glutamic acid (Glu), glycin (Gly), isoleucine (Ile), leucine (Leu), proline (Pro),
234 serine (Ser), threonine (Thr), tyrosine (Tyr) and valine (Val) were detected. Whereas
235 arginine (Arg), histidine (His), lysine (Lys), methionine (Met) and phenylalanine (Phe)
236 were not detected. However, with increased Al, Met, Phe, Arg, Lys and His became
237 detectable in succession and with the exception of Met and Arg, increased gradually,
238 while the release of Cys, Leu, Val and Pro decreased simultaneously. When the external
239 Al was in excess of 300 μM, Ser, Glu, Cys and Asp were undectable. But, the
240 Al-triggered release of aromatic Tyr and Phe rose steeply with Al concentrations.

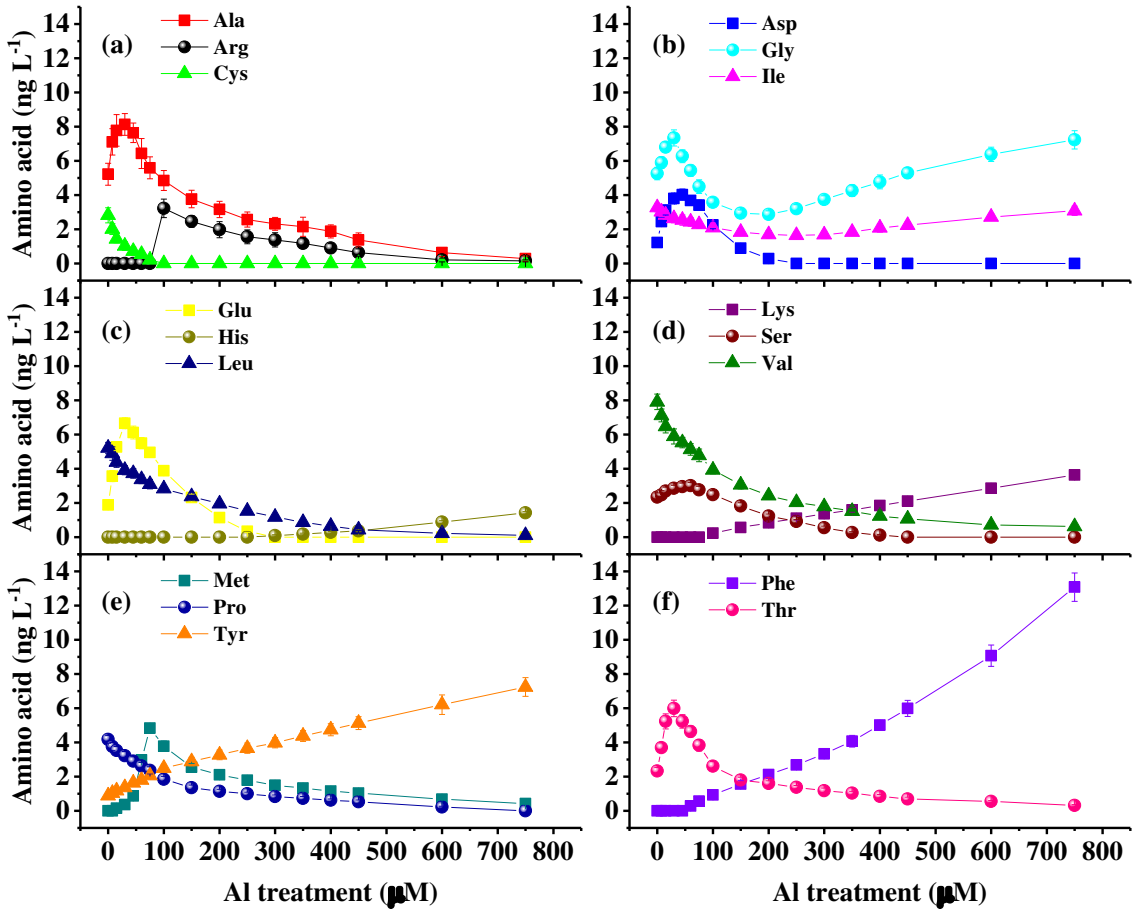

Fig. 2 Al-stimulated amino acids released from Masson pine roots. Herein, (a) for Ala (alanine), Arg (arginine) and Cys (cystine); (b) for Asp (asparagine), Gly (glycin) and Ile (isoleucine); (c) for Glu (glutamic acid), His (histidine) and Leu (leucine); (d) for Lys (lysine), Ser (serine) and Val (valine); (e) for Met (methionine), Pro (proline) and Tyr (tyrosine); (f) for Phe (phenylalanine) and Thr (threonine). Error bars represented SD from n = 9 replicates.

Secondary metabolites

The results presented in Fig. 3 indicated that the release of secondary metabolites was greatly influenced by acidic rhizospheric Al. In the blank assay, cyclohexanol, cyclohexanone, 6-methyl-2-methyl-dicyclo(3,1,1)heptane, methylnaphthalene, 2,6-ditert-butylphenol, 2-ethyl-1,3-dimethylbenzene, 1,2,3,4-tetramethylbenzene, 10-methyl-methyl hendecylate, β-phellandrene and n-dotriacontane, were released from

the pine roots. However, in the Al-treated rhizosphere, detectable secondary metabolites were: cyclohexanol, cyclohexanone, methylnaphthalene, 2-ethyl-1,3-dimethylbenzene, 10-methyl-methyl hendecylate, n-dotriacontane, 3-methyl-furandione(2,5), N,N'-ethyldiglycin, 1,2,3,5-tetramethylbenzene, pentanal, pentadiene-(1,4), 5-nitrylpyrazole, cyclobutanol-6-methylheptyl amine-2 and 2-nitryl-1-caprolene-4-alkyne. With increasing rhizosphere Al^{3+}, the eight secondary metabolites gradually disappeared. Simultaneously, the eight new secondary metabolites were successively released. When Al-treated concentration was 750 μM, only cyclohexanol, cyclohexanone, and 2-nitryl-1-caprolene-4-alkyne were detected.

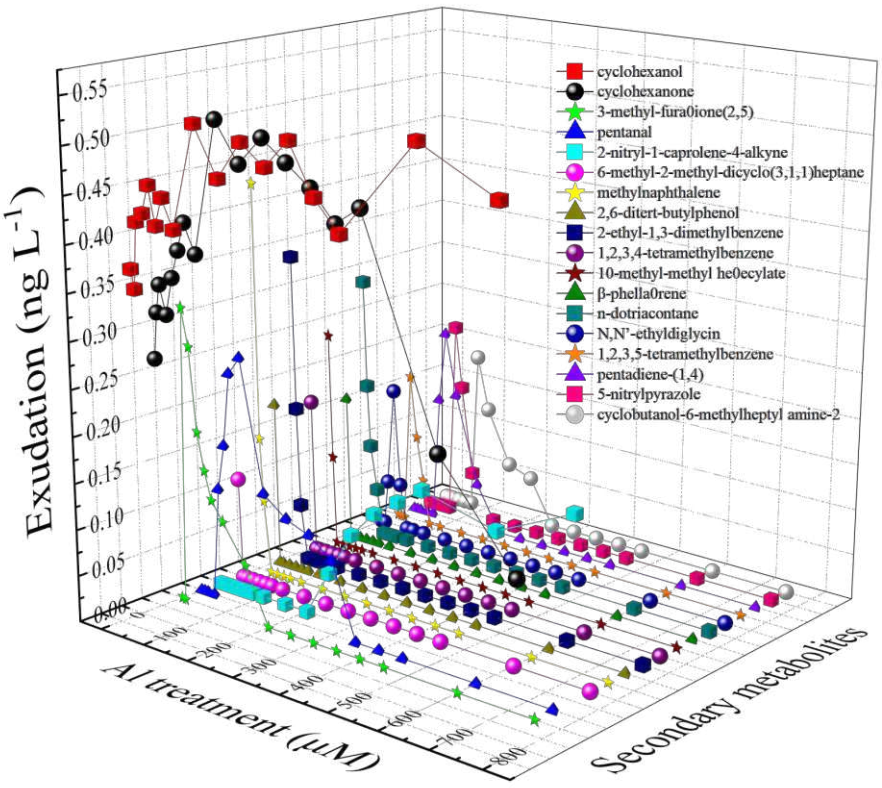

Fig. 3 Effects of rhizosphere Al on the secondary metabolites (ng L^{-1}) exuded from Masson pine roots. The data shown in Fig. 3 was the mean and standard deviation (SD) of nine replicated treatments.

Al-triggered influence on the pine roots

Endogenous hormones

The endogenous hormones, abscisic acid (ABA), gibberellic acid (GA_3), indole-3-acetic acid (IAA) and zeatin riboside (ZR), were detected using ASE-SPE-LC-ESI-MS/MS methodology (Wang et al. 2016). As shown in Fig. 1d, a higher level of ABA in the pine roots was observed in the positive response to increasing external Al. In contrast, the Al-induced negative response of GA_3 was observed to increasing external Al, its maximum concentration occurred at zero Al treatment. For IAA and ZR, they seem have no effect on the Al detoxicity of pine roots. Their levels were characterized by ZR > IAA throughout the experiment.

Root dry weight and accumulated Al

Figure 4a demonstrated that Masson pine root growth was inhibited by increasing Al concentrations. Accurately, added Al had a negative effect on the root dry weight. The linear equation used for the estimations of root dry weight was as follows:

$$Wt = 0.255 - 2.38E^{-4} \times C_{Al} \qquad R^2 = 0.983 \qquad P < 0.0001 \quad \text{(Eq. 1)}$$

where Wt is the dry weight of pine root (mg g^{-1}dw), and C_{Al} is the Al-treated concentration (μM).

Meanwhile, added Al had a positive effect on the Al contents accumulated in the pine roots (Fig. 4b). When Al-treated concentration was more than 300 μM, the Al accumulated contents increased gradually from 2.5 to 8.3 μg g^{-1}dw. The Al accumulated contents can be calculated as follows:

$$Q_{Alac} = 0.104 + 0.0096 \times C_{Al} \qquad R^2 = 0.985 \qquad P < 0.0001 \quad \text{(Eq. 2)}$$

where Q_{Alac} is the Al content accumulated in the pine roots (μg g^{-1}dw), and C_{Al} is the Al-treated concentration (μM).

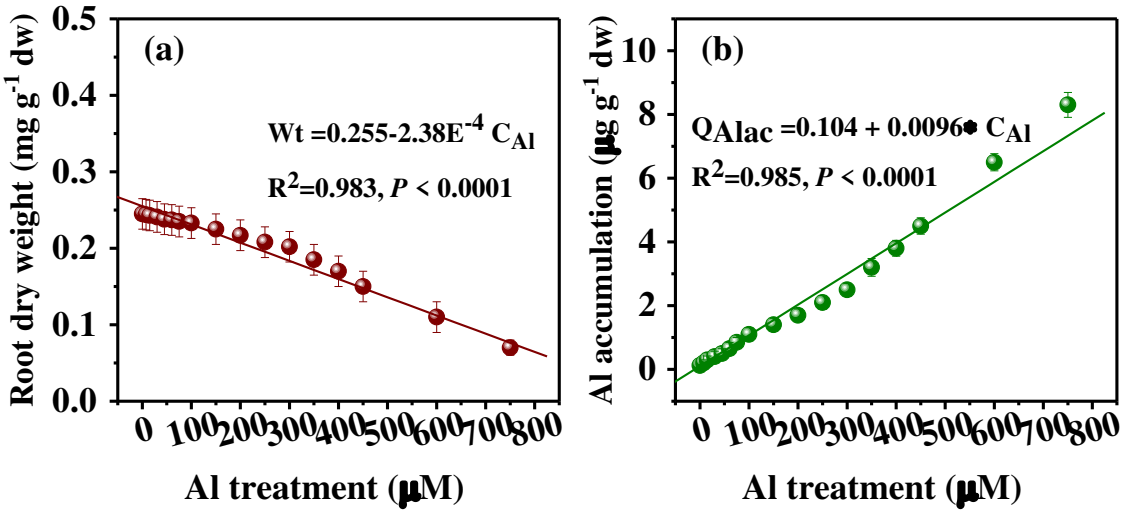

Fig. 4 Al-stimulated variations of root dry weight (a) and the Al accumulated in Masson pine roots (b). Error bars represent SD from n = 9 replicates.

Discussion

An increased understanding of the Al-tolerant rhizosphere processes can help in the growth of Masson pine that is adapted to acidic soils (Wu et al. 2009). Our work emphasizes Masson pine's response to rhizosphere Al.

Al-stimulated root-released compounds are a useful system for studying how the Al signal expresses physiological responses underlying Al tolerance, and we believe that their compositional changes play a significant role in the transduction of Al signals in the root apex of Masson pine. In general, plants may produce more root-released compounds under environmental stress (Hinsinger et al. 2005). The root-released compounds mainly were electrolytes, H^+, sugar, organic acids, amino acids and other secondary metabolites.

We really want to perform a comprehensive analysis of the root-released compounds. Due to the limitation of instrument sensitivity, we could only detect these compounds at present, although we try to figure out every peak detected via our analytical instruments including HPLC, GC-MS and LC-MS. Several studies also support a mechanism whereby in the plant-soil interface, active Al ions chelate with root-released organic compounds to alleviate Al toxicity (Eticha et al. 2005). However, the specific mechanisms of Al toxicity are still poorly understood in tree species. Following is the discussion on the characterization of Al toxicity for Masson pine, which will benefit the understanding of the Al-tolerant mechanism of Masson pine.

Variation of rhizospheric pH

Evidence also exists to show that rhizosphere pH is primarily caused by root and microbial respiration, unbalanced uptake of inorganic anions and cations, release of organic anions and oxidation of soil minerals (Hinsinger et al. 2005). This Al-induced acidification possibly occurs as a consequence of differential rates in the uptake of cations and anions by Masson pine roots. The excess of H^+ to counterbalance a lack uptake of Al over anions (mainly OH^-) has caused a rapid decrease in rhizosphere pH (0.58 pH, Fig. 1a). The ability to acidify the root medium may be genetic, relating to physiological adaptation and Al tolerance (Haruta and Constabel 2003). It was found that Al exposure elicited changes both in root organic acid content and rhizosphere H^+ release (Zhang et al. 2019). Acidification at the root surface increases the activity of rhizotoxic Al ions, which might partly affect the toxic effects of Al on tree species (Hirano et al. 2012; Rehmus et al. 2014). This effectiveness of rhizosphere acidification in alleviating Al toxicity has also been demonstrated by root release of oxalic acid which can chelate

328 and detoxify excessive Al in the apoplasm or rhizosphere (Liang et al. 2013). Thus,
329 rhizosphere pH can negatively affect the activity of phytotoxic Al ions, which might
330 partly alleviate the toxic effects of Al on tree species.

331 Sugar and organic acids released from the pine roots

332 The results in Fig. 1b showed that the addition of Al had a positive effect on the secretion
333 of glucose. In contrast, the concentrations of fructose and aldose were very low during
334 the entire experimental assay. The relatively high Al-stimulated release of glucose can be
335 explained by the fact that Al affects adaptive reactions relating to carbon metabolism.
336 Root-released glucose may be perhaps related to selective permeability of the plasma
337 membrane. The root-released organic acids are another observed physiological change in
338 response to added Al. The results in Fig. 1c showed that Al quantitatively stimulates the
339 efflux of oxalic acid from excised root apices of the pine seedlings. Clearly, organic acids
340 have been directly implicated in a number of rhizosphere processes such as
341 Al-detoxification and nutrient solubilization by roots. The Al-induced secretion of oxalic
342 acid has been reported as an Al-tolerance mechanism by Li et al. (2000). Their direct role
343 in these rhizosphere processes, however, has been difficult to establish due to the many
344 interdependent factors influencing the release of organic acids. These factors include
345 solid phase sorption/desorption reactions, metal complexation reactions, leaching and
346 microbial degradation (Ramesh et al. 2018). The present results confirm that in Masson
347 pine, as in other herb, the release of an Al chelator might partly alleviate the toxic effects
348 of Al on tree species.

349 Amino acids and secondary metabolites released from the pine roots

Rhizosphere Al affected amino acid secretion, making several original amino acids disappear and some new amino acids appear (see Fig. 2). This indicated that Al-triggered release of amino acids was different from the results of controls. Interestingly, Al exposure triggered much a small stimulation in certain amino acids and cause severe physiological disorders in Masson pine. We obtained 17 dose-response curves relating root apical amino acids release to Al activity. These responses were interpreted as the result of Al-induced environmental stress.

The composition of secondary metabolites released from the roots was different in the presence of Al. Some original secondary metabolites detected in the blank assay were not, however, observed under condition of Al treatments, while there were some new secondary metabolites to be later found. What is the difference between original secondary metabolites and new secondary metabolites? These new secondary metabolites are an interesting response to the addition of Al, having developed strategies to avoid or tolerate Al-induced effects, and progressively stimulate Masson pine to grow under Al enhanced conditions. The increased root-released metabolites can be explained by the disorganization of the physiological functions of fine roots that cannot prevent the leaking out of sugars, amino acids and other important compounds, such as secondary metabolites (Bourgaud et al. 2001). More information has to be included about transporters and ion channels, which could give hints on the release of organic secondary metabolites from the roots. Chen et al. (2006) revealed that these molecules contribute to plant fitness by interacting with the ecosystems.

Endogenous hormones in the pine roots

As shown in Fig. 1d, the rooting response of ABA, GA_3, IAA, and ZR to increased levels of Al ion was examined. Preliminary studies indicate that these endogenous hormones are regulators produced by plants themselves, as a minor component of the metabolome, which control the physiological processes and are of particular significance given their role in the protective responses of plants against stress (Haruta and Constabel 2003). During plant growth, ABA, as a stress hormone, has been shown to play a central role in adaptive responses to environmental stress (Zhang et al. 2018). We obtained a direct relationship between these detected hormones and high-Al tolerance. The significant variation of ABA and GA_3 in Al-treated roots seems to be associated with Al response, but the opposite responses to Al were observed for ABA and GA_3. This possibility of the interpretation will be the focus of future work in our laboratory.

Absorption of Al by Masson pine roots and root growth

Al accumulation in Masson pine roots under normal growth conditions was relatively low, with an average concentration of 0.1 μg g^{-1}dw. Most trees contain no more than 0.2 μg g^{-1}dw of Al (Zhang et al. 2014). The Al^{3+} accumulated in root systems is influenced by many factors, such as transpiration (the rate of moisture absorbed by roots), coefficient of ionic diffusion (ionic migration and solubility), concentration gradient, and other ions in the root system (Kopittke et al. 2015). The Al influx finally reaches the root system, and is accumulated in the pine roots, around the cortex. The initial and most dramatic symptom of Al accumulated in roots results in a reduced root system (Mossor-Pietraszewska 2001). Long-term exposure to Al, such as 100 days, will lead to nutrient deficiencies, mainly of P, K, Ca and Mg, and then inhibition of root growth

394 generally (Vitorello et al. 2005; Schaberg et al. 2006; Zhang et al. 2019). Barceló and
395 Poschenrieder (2002) observed that root growth is immune to lower level additions of Al.

396 Inferred from the significant decrease of root dry weights at Al levels from 300 μM
397 to 750 μM (Fig. 3a), these pine seedlings were undergoing severe Al stress. Although
398 there is no direct link between changes in the rooting Al accumulation and alleviation of
399 root growth inhibition, our interpretation is that higher Al strongly impacts root cell
400 membrane integrity and favors the leak of root cell solutes, which results in obvious
401 decreases in the root growth (Kopittke et al. 2015; Zhang et al. 2018).

402 Mechanisms underlying Al-tolerance based on this Masson pine case-study

403 (1) Inhibition of root growth is a well-known effect of Al toxicity, and root tips have
404 been suggested as a primary site for Al-induced injury in plants. The rhizosphere
405 response to increased levels of Al in a hydroponic setup behaved differently in the
406 composition of root-released compounds, root growth inhibition and rooting Al
407 accumulation. Al resistance is related to rhizosphere Al ion concentrations and is
408 characterized by Al exclusion from the root tip, changes in rhizosphere pH, and increased
409 release of organic acids (Imadi et al. 2016).

410 (2) The pine seedlings emitted different physiological signal in response to Al
411 exposure in their rhizosphere (Wu et al. 2009). Many compounds released from the pine
412 roots (such as electrolyte, H^+, sugar, organic acids, amino acids, endogenous hormones,
413 enzymes and other secondary metabolites) react or balance with external Al ions to
414 alleviate Al toxicity. The composition of root-released compounds is related to
415 Al-resistance (Hinsinger et al. 2005). The observed Al-induced changes in the

root-released compounds are a response to Al that could contribute to alleviate Al toxicity.

(3) Finally, Al stimulates the efflux of oxalic acid by activation of anions channels (Piñeros et al. 2002). Activation of anion efflux channels facilitating the efflux of oxalic acid seems responsible for Al resistance (Poschenrieder et al. 2008). The Al-induced organic acid anions are transported through the specific anion channel across the plasma membrane into plant rhizosphere, which makes the plasma membrane positively charged and rejects external Al^{3+} (Ohno et al. 2003) as well as H^{+} (Zhang et al. 2019). The oxalic acid stained inside root tip may react with smaller amounts of Al. These complexes are not toxic to plant cells (Wenzl et al. 2002). Outside Masson pine roots, the secreted oxalic acid reacts or balances with external A1 to obtain Al tolerance (Wang et al. 2020). The stable Al-organic acid complexes do not across rooting plasma membrane (Kinraide et al. 2005) and cannot be accumulated the pine roots, which could contribute to alleviate Al toxicity.

Phytotoxic effects of Al in acidic soil on Masson pine destroy many physiological and biochemical pathways occurring in roots, and results in Al uptake and exudation of various compounds (Imadi et al. 2016; Yang et al. 2017). Herein, this root/rhizospheric responses to Al-toxicity were observed comprehensively. We anticipate that our experimental data-set will further enhance our understanding of the bigger picture about Al-toxicity/Al-tolerance/Al-adaptation of forests impacted by acidification.

Conclusions

In summary, the present study using a hydroponic technique revealed the characterization of Al toxicity for Masson Pine through comprehensive laboratory dose-gradient experiments. Here, we observed that Al tolerant species tend to acidify their apoplast (rhizosphere pH from 4.50 to 3.92). Higher Al-treated concentrations at low pH contributed to changes in the composition of root-released compounds (oxalic acid, sugars, amino acids, endogenous hormones and secondary metabolites), Al uptake and accumulation, root growth of Masson pine cultivar. The pine root defense system gradually shows an inheritable tolerance to a broad range of rhizosphere Al concentrations. The observed dose-responses of root-released compounds to Al could contribute to alleviate Al toxicity. It is possible to derive dose-response models to calculate thresholds for Al toxicity based on growth or changes in metabolic profiles. Our results highlight the importance of rhizospheric release and regulatory processes, which may play an important combined role in regulating Al-resistance of *Pinus nassoniana*, as for how they modulated these rhizospheric processes is in progress. Additionally, exposure to Al also influences H^+-adenosine triphosphatase (H^+-ATPase) activity in Masson pine (Minorsky 2019). The biochemistry of Al and the H^+-ATPase mechanisms by which it affects Al tolerance need to be investigated in future.

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