



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

Ping Wang<sup>1</sup> · Sijie Zhou<sup>1</sup> · Ao Li<sup>1</sup> · Linbei Xie<sup>1</sup>

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## Abstract

Trees in general are very tolerant of aluminum (Al, mainly  $\text{Al}^{3+}$  at  $\text{pH} \leq 5.0$ ), and the small effects seen in the contaminated soils may mislead people that the contamination is unimportant. The key point of this study was to characterize the Al toxicity for Masson pine (*Pinus massoniana* Lamb). The objectives were to discover the specific eco-physiological relationship between pine root growth and rhizosphere Al, and to investigate the Al effects on the root-released compounds of sugars, organic acids, amino acids, secondary metabolites, as well as rhizosphere pH and endogenous hormones. Masson pine seedlings were cultivated on a hydroponic setup. Through comprehensive dose-gradient experiments, the Al-triggered root-released compounds 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 glucose, and also could accelerate 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 100  $\mu\text{M}$ . Greater additions of Al (> 300  $\mu\text{M}$ ) 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. Four endogenous hormones were also determined in the pine roots. Gibberellic acid ( $\text{GA}_3$ ) decreased, whereas abscisic acid (ABA) simultaneously increased with the addition of Al. Their inflexional concentrations were most frequently observed at 100  $\mu\text{M}$ , 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 could trigger pine roots to release the sugars (glucose, fructose + aldose), organic acids (oxalic acid, and malic acid), amino acids, secondary metabolites, and endogenous hormones. Meanwhile exposure of growing root apices to toxic Al concentrations inhibited 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.

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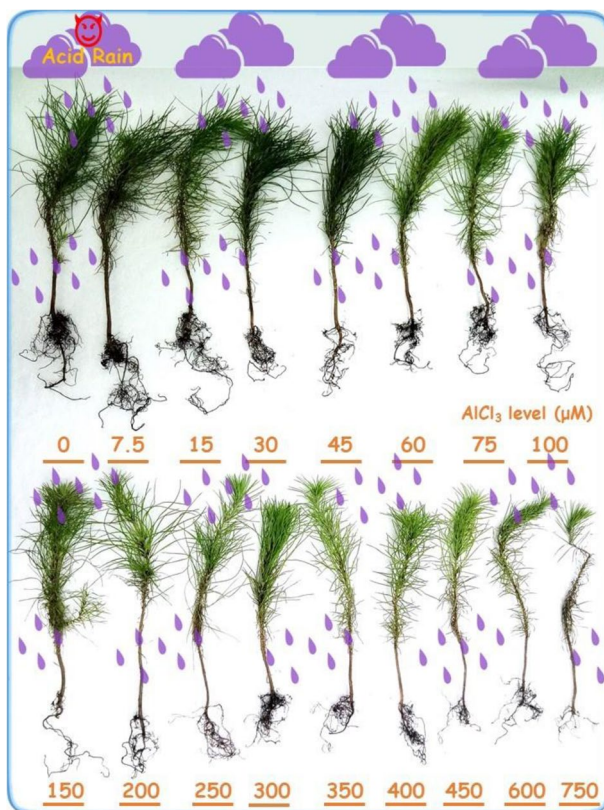
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✉ Ping Wang  
18061713169@163.com

<sup>1</sup> School of Biology and the Environment, Nanjing Forestry University, Nanjing 210037, People's Republic of China

## Graphical abstract



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

## 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; Yao et al. 2020). 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  $\text{Al}^{3+}$ ) may threaten the integrity of forest ecosystems as a result of acid deposition. Acidification may lead to forest soil degradation, affecting the soil functions. The  $\text{Al}^{3+}$  released into soil solution is usually well below  $50 \mu\text{M}$  at  $\text{pH} > 5.5$ , but rises 100-fold at  $\text{pH} 4.5$ , which is of risk for the growth of Al-sensitive plant species (Wang et al. 2006; Yang et al. 2015). High Al concentration may disrupt plant root functions and the metabolic changes associate with root-released compounds. The root system, especially root apex, is the critical site for Al

toxicity. Yao et al. (2020) also observed Al resistance in the root tips of Masson pine. Normally, plant roots excrete low molecular weight organic molecules into plant rhizosphere to adapt various stressful circumstances (Wenzl et al. 2002). Study of the root-released compounds holds great promise for revealing the phytotoxic effects of Al on tree rhizosphere.

Rhizosphere is an important root-soil interface for releasing organic compounds, intense nutrients exchange and microbial activity, and also a gateway for potentially toxic pollutants such as Al, in which normal root physiological action is greatly influenced by root-released compounds (Yang et al. 2019; Gomez-Zepeda et al. 2021). For example, Riaz et al. (2018) and Yang et al. (2020) reported that the efflux of low molecular weight organic anions from root apices could protect the root by chelating and thus detoxifying Al in the rhizosphere. Yan et al. (2019) reviewed immobilization of the cell wall, selective permeability of the plasma membrane, formation of rhizosphere pH barrier and evolution of Al-tolerant enzymes in higher plants under Al stress. The study by Pandey et al. (2013) also confirmed that salicylic acid, as a phytohormone, could alleviate Al toxicity

in rice seedlings by reducing Al uptake and accumulation, suppressing oxidative damage and increasing antioxidative defense. Therefore, the mechanism and effects of the root exudation from Masson pine treated with acidic Al deserves serious attention.

Numerous Al toxicity mechanisms have been proposed (Wang et al. 2006; Imadi et al. 2016). In woody species, Al detoxification may be achieved by the combination of organic anions secretion from roots and intracellular Al chelation with organic anions. Putative exclusion mechanisms proposed include binding of Al in the cell wall, an Al-induced rhizosphere pH barrier, and root-released Al-chelating compounds (Liang et al. 2013; Yang et al. 2017). Organic root-released compounds have been suggested to play a crucial role both in Al exclusion, via release from plant root, and Al detoxification in the apoplast or rhizosphere, where low molecular weight organic anions could chelate Al and reduce or prevent its toxic effects at the cellular level (Yang et al. 2019).

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. 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  $\mu\text{M}$ , based on our earlier study (Wang et al. 2006, 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.

## 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 mineral compounds was prepared (in  $\mu\text{M}$ ): 250  $\text{NH}_4\text{NO}_3$ , 60  $\text{KH}_2\text{PO}_4$ , 220  $\text{K}_2\text{SO}_4$ , 188  $\text{CaCl}_2$ , 62  $\text{MgSO}_4$  and 95  $\text{Fe-EDTA}$ , 46  $\text{H}_3\text{BO}_3$ , 0.3  $\text{CuSO}_4$ , 0.1  $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$ , 9.2  $\text{MnSO}_4$ , 0.8  $\text{ZnSO}_4$  (van Schöll et al. 2004). The mixed solution was divided into 17 equal parts (20 L). In the soils of Chongqing, Al concentrations were 55–146  $\mu\text{M}$  from 2006 to 2015 (Wang et al. 2020). To better observe the effects of Al stress, the experimental Al concentrations

were selected in the range of 0 to 750  $\mu\text{M}$ . Different amounts of  $\text{AlCl}_3$  (0, 0.0200, 0.0400, 0.0800, 0.1200, 0.1600, 0.2000, 0.2667, 0.4000, 0.5334, 0.6667, 0.8000, 0.9334, 1.0667, 1.2001, 1.6001, and 2.0001 g) were respectively 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  $\mu\text{M}$ . Then the pH values of these solutions were initially adjusted to 4.5 using 1%  $\text{HCl}$  or 1%  $\text{NaOH}$ , and subsequently checked and adjusted again three times a week. The nutrient solution without Al was used for control assay. The solution was aerated by pump, which connected the pot with pump line. The seedlings from each treatment were harvested after 100 days 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. There were two sterilization protection measures to be implemented. (1) The nutrient solutions were changed regularly every 10 days. (2) Ultraviolet radiation ( $\lambda = 365 \text{ nm}$ ) was used to disinfect the growth chamber, pots and nutrient solutions prior to use.

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

The root-released compounds were collected following the procedures described in our previous studies (Wang et al. 2006, 2015b). Briefly, the roots were treated with deionized water, and then exposed to a 1 L 0.5 mM  $\text{CaCl}_2$  solution (pH 4.5) with corresponding Al level for 24 h and then washed with 100 mL of deionized water. 25 seedlings per pot were one measurement. The 9 replicate measurements about 9 pots were carried out for each Al treatment and an “average value  $\pm$  standard deviation (SD)” is reported. To avoid interaction between Al and other nutrients such as P, a simple salt solution containing 0.5 mM  $\text{CaCl}_2$  was used as the basal treatment. The above-mentioned Al treatment solution was placed on a shaker, centrifugally separated (60 rpm) for 2 h, and filtered. The filtrate was divided into two equal parts. One part was used to measure pH, and the other part was concentrated to 50.00 mL under vacuum and analyzed for sugars, amino acids, organic acids and secondary metabolites. The sample pretreatment was performed at  $25 \pm 1 \text{ }^\circ\text{C}$ .

## Analysis

### Rhizosphere pH

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

### Sugar

A 5.00 mL filtrate was hydrolyzed with 10.00 mL of 4% H<sub>2</sub>SO<sub>4</sub> under vacuum at 110 °C for 1 h. After cooling, the hydrolysate was washed with deionized water, filtered (Whatman No.2, USA) and dried at 60 °C (also under vacuum) by a rotary evaporator. The dried sample was then dissolved in 5 mM H<sub>2</sub>SO<sub>4</sub>. The sugar (monosaccharide) in the hydrolysate was separated and quantified by injecting 10 µL into a HPLC (Waters 600, USA) with RI×4 detector, equipped with a Sugar-pak™ 1. P/N 85,188 column (Waters, USA). The column temperature was 85 °C. Milli-Q water was used as mobile phase with a flow rate of 0.6 mL min<sup>-1</sup>.

### Organic acids

A 5.00 mL filtrate was passed through a cation exchange column (16×14 mm) filled with 5 g of Amberlite IR-120B resin (H<sup>+</sup> form, Shanghai Chemical Reagent Co., China), followed by an anion-exchange column (16×14 mm) filled with 2 g of Dowex 1×8 resin (100–200 mesh, format form; Shanghai Chemical Reagent Co., China). The organic acids retained on anion-exchange resin were eluted by 1 M HCl, and the eluate was concentrated. 10 µL concentrating solution was injected onto an Aminex HPX-87H column (7.8 mm i.d.×300 mm, 9 µm). The determination of organic acids was carried out with electrospray ionization-tandem mass spectrometry (ASE-SPE-LC-ESI-MS/MS). The mobile phase used was 5 mM H<sub>2</sub>SO<sub>4</sub> at a flow-rate of 0.5 mL min<sup>-1</sup>. Detection was at a wavelength of 210 nm. Column temperature was 50 °C (Wang et al. 2015a).

### Amino acids

A 5.00 mL filtrate was hydrolyzed with 8.00 mL of 6 M HCl under vacuum at 110 °C for 24 h. After cooling, the hydrolysate was washed with deionized water, filtered (Whatman No.2, USA) and dried at 60 °C (under vacuum) by a rotary evaporator. The dried sample was then dissolved in 0.01 M HCl. The amino acids in the hydrolysate were separated and quantified by injecting 50 µL into a Hitachi 835-50 Amino Acid Automatic Analyzer (Hitachi, Japan) equipped with a 2.6 mm×150 mm ion exchange column coated with resin 2619<sup>#</sup>. The column temperature was 53 °C. Sodium citrate buffers (pH 6.3) were used as eluents with a flow rate of

0.225 mL min<sup>-1</sup>. The light absorbance of the amino acids was detected with a 166 Detector (Beckman Instruments) at 570 nm.

### Secondary metabolites

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

### Root dry weight and accumulated Al

The Masson pine roots were treated with deionized water (25 seedlings per treatment) and cut into small pieces, which were dried at 70 °C for 48 h to determine their dry weight. The dried roots were weighed, ground, acid-digested, filtered, and finally concentrated to 50.00 mL. The total Al was determined by inductively coupled plasma atomic emission spectrometer (ICP-AES, PS-1000AT, USA) (Wang et al. 2012).

### Endogenous hormones

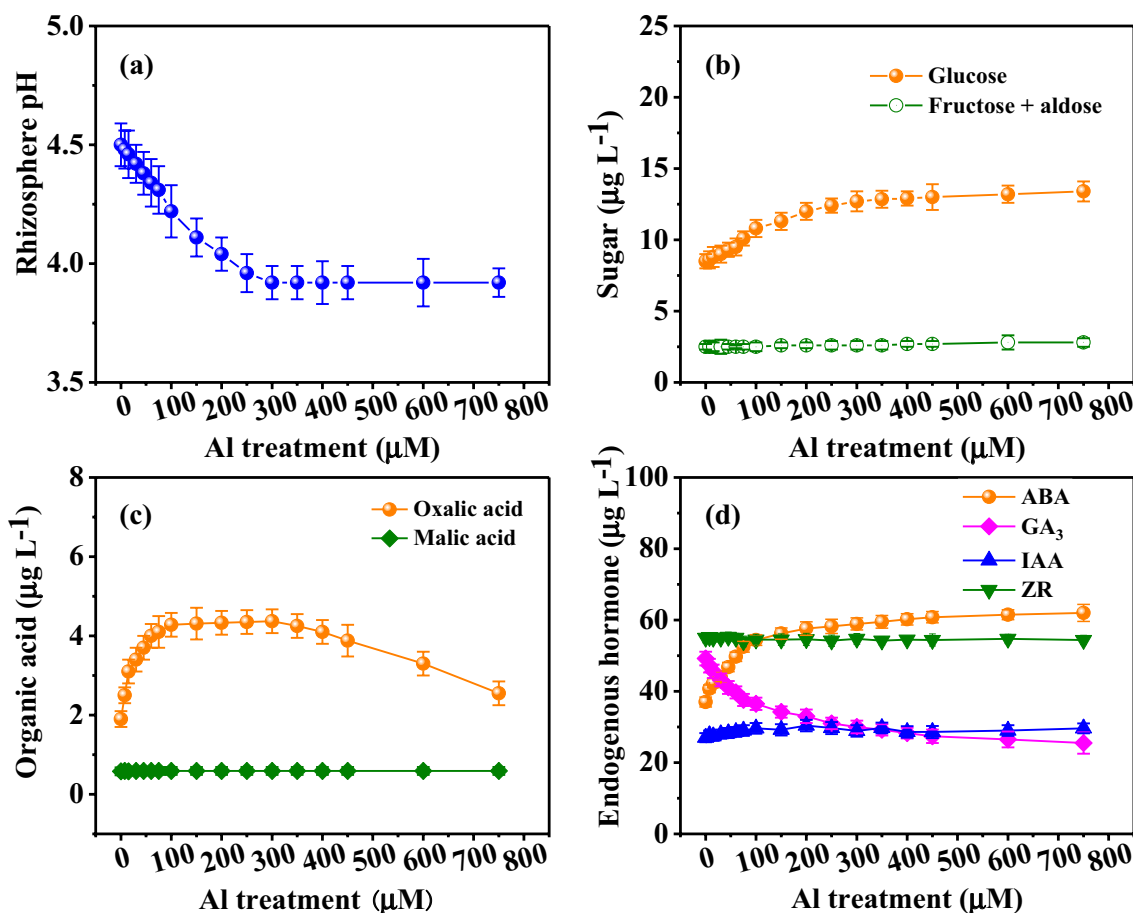
Endogenous hormones were analyzed using a LC-ESI-MS/MS system. 10 µL of the above-mentioned solution for Al determination was injected onto a KC-811 column. Detection was at a wavelength of 254 nm. The mobile phase used was a mixed solution (methanol/water/acetic acid 50:49.3:0.7, V/V/V) at a flow rate of 0.6 mL min<sup>-1</sup>. Column temperature was 35 °C. Quantification was based on the LC-ESI-MS/MS peak area found for the base peak of single hormone (Wang et al. 2016).

### Statistical analysis

The data presented in Figs. 1, 2, 3, 4 were the mean and SD of nine replicated measurements (one measurement for one pot). 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 Figs. 1, 2, 3, 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



**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 measurements ± SD (standard deviation). Error bars represent SD from n=9 replicates

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

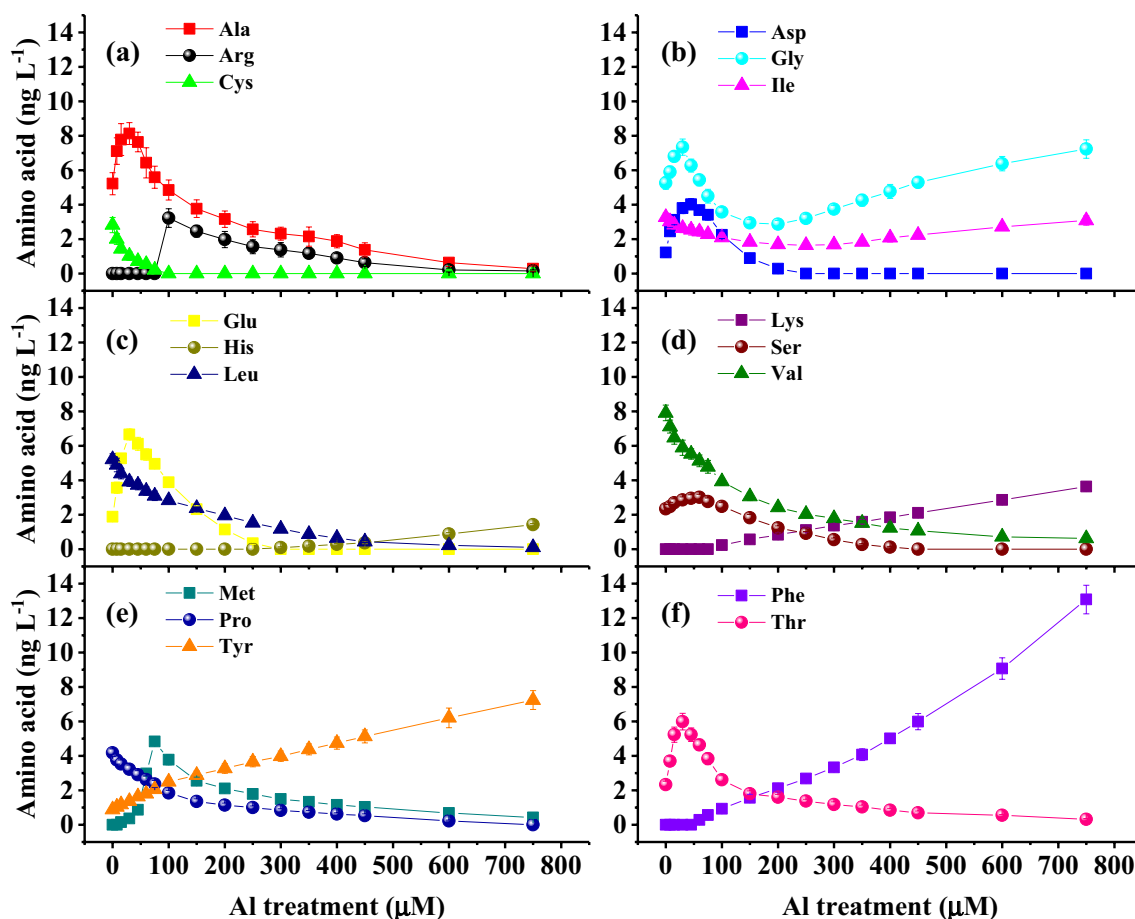
The variation of Al-contaminated rhizosphere pH was assessed in the presence and absence of Al (Fig. 1a). At lower Al level (100 μM), the addition of Al caused the rhizosphere pH to decrease from 4.50 to 4.22. However, when Al level varied from 100 to 300 μM, the rhizosphere pH decreased rapidly from 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.

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

#### Organic acids

Oxalic and malic acids were determined using ASE-SPE-LC-ESI-MS/MS system (Wang et al. 2015a). The



**Fig. 2** Al-stimulated amino acids released from Masson pine roots. Herein, **a** for Ala (alanine), Arg (arginine) and Cys (cysteine); **b** for Asp (aspartic acid), 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 represent SD from  $n=9$  replicates

occurrence of Al-induced oxalic acid in the Masson pine rhizosphere and its special relevance concerning Al levels were presented in Fig. 1c. Increasing rhizosphere Al from 0 to 100  $\mu\text{M}$  slightly accelerated the release of oxalic acid. Interestingly, high  $\text{Al}^{3+}$  exposure ( $> 300 \mu\text{M}$ ) triggered a significant small inhibition in oxalic acid. In contrast, there was no clear varying trend for the root release of malic acid.

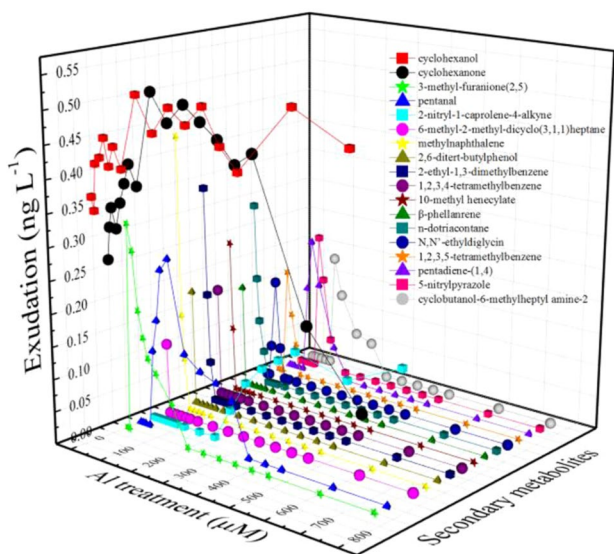
### Amino acids

The effects of rhizosphere Al on the root-released amino acids were presented in Fig. 2. In the blank assay (without Al), twelve amino acids, including alanine (Ala), cysteine (Cys), aspartic acid (Asp), glycin (Gly), isoleucine (Ile), glutamic acid (Glu), leucine (Leu), serine (Ser), valine (Val), proline (Pro), tyrosine (Tyr), and threonine (Thr) were detected. Whereas arginine (Arg), histidine (His), lysine (Lys), methionine (Met) and phenylalanine (Phe) were not detected. However, with increased Al, Arg, His, Lys, Met,

and Phe became detectable in succession and with the exception of Arg and Met, increased gradually, while the release of Cys, Leu, Val and Pro decreased simultaneously. When the external Al was in excess of 300  $\mu\text{M}$ , Cys, Asp, Glu and Ser were undetectable. But, the Al-triggered release of aromatic Tyr and Phe rose steeply with Al concentrations.

### 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, methyl-naphthalene, 2,6-ditert-butylphenol, 2-ethyl-1,3-dimethylbenzene, 1,2,3,4-tetramethylbenzene, 10-methylhendecylate,  $\beta$ -phellandrene and n-dotriacontane were released from the pine roots. However, in the Al-treated rhizosphere, detectable secondary metabolites were: cyclohexanol, cyclohexanone, 3-methyl-furandione(2,5),



**Fig. 3** Effects of rhizosphere Al on the secondary metabolites (ng L<sup>-1</sup>) exuded from Masson pine roots. The data shown in Fig. 3 is “mean ± SD” of nine replicated measurements

pentanal, 2-nitryl-1-caprolene-4-alkyne methylnaphthalene, 2-ethyl-1,3-dimethylbenzene, 10-methyl hendecylate, n-dotriacontane, N,N'-ethylidiglycin, 1,2,3,5-tetramethylbenzene, pentadiene-(1,4), 5-nitrylpyrazole, and cyclobutanol-6-methylheptyl amine-2. With increasing rhizosphere Al<sup>3+</sup>, the eight secondary metabolites (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 hendecylate, β-phellandrene, n-dotriacontane) gradually disappeared. Simultaneously, the eight new secondary metabolites (3-methyl-furandione(2,5),

pentanal, 2-nitryl-1-caprolene-4-alkyne, N,N'-ethylidiglycin, 1,2,3,5-tetramethylbenzene, pentadiene-(1,4), 5-nitrylpyrazole, cyclobutanol-6-methylheptyl amine-2) were successively released. When Al-treated concentration was 750 μM, only cyclohexanol, cyclohexanone, and 2-nitryl-1-caprolene-4-alkyne were detected.

### Al-triggered influence on the pine roots

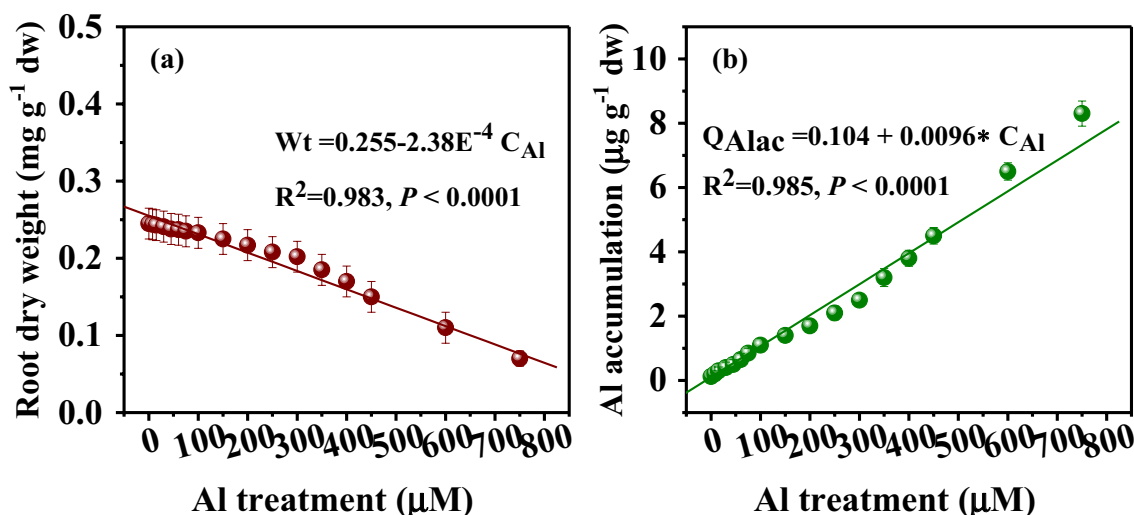
#### Endogenous hormones

The endogenous hormones, abscisic acid (ABA), gibberellic acid (GA<sub>3</sub>), 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, 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<sub>3</sub> 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 estimating root dry weight was as follows:

$$Wt = 0.2552.38E^{-4} \times C_{Al} \quad R^2 = 0.983P < 0.0001 \quad (1)$$



**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

where  $W_t$  is the dry weight of pine root ( $\text{mg g}^{-1}\text{dw}$ ), and  $C_{\text{Al}}$  is the Al-treated concentration ( $\mu\text{M}$ ).

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  $300 \mu\text{M}$ , the Al accumulated contents increased gradually from 2.5 to  $8.3 \mu\text{g g}^{-1}\text{dw}$ . The Al accumulated contents can be calculated as follows:

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

where  $Q_{\text{Alac}}$  is the Al content accumulated in the pine roots ( $\mu\text{g g}^{-1}\text{dw}$ ), and  $C_{\text{Al}}$  is the Al-treated concentration ( $\mu\text{M}$ ).

## Discussion

An increased understanding of the Al-tolerant rhizosphere processes can help in the growth of Masson pine that is adapted to acidic soils (Yao et al. 2020). 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. Generally, plants may produce more root-released compounds under environmental stress, the changes in their contents were related to the expression of related metabolic genes (Riaz et al. 2018; Yang et al. 2020). The root-released compounds mainly were electrolytes,  $\text{H}^+$ , sugar, organic acids, amino acids and other secondary metabolites. We really want to perform a comprehensive analysis of the root-released compounds under acidic Al stress. 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, ICP-AES 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 (Yan et al. 2019). 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 (Yang et al. 2019; Yao et al. 2020). 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  $\text{H}^+$  to counterbalance a lack uptake of  $\text{Al}^{3+}$  over anions (mainly  $\text{OH}^-$ ) 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 (Liu et al. 2016; Yang et al. 2020). It was found that Al exposure elicited changes both in root organic anions and rhizosphere  $\text{H}^+$  release (Zhang et al. 2019a, b). Acidification at the root surface increases the activity of rhizotoxic Al ions, which might partly affect the phytotoxic effects of Al ions on tree species (Hirano et al. 2012; Rehmus et al. 2014; Yao et al. 2020). This effectiveness of rhizosphere acidification in alleviating Al toxicity has also been demonstrated by root release of oxalic acid which can chelate and detoxify excessive Al ions in the apoplasm or rhizosphere (Liang et al. 2013; Yao et al. 2020). Thus, rhizosphere pH can negatively affect the activity of phytotoxic Al ions, which might partly alleviate the toxic effects of Al on tree species.

### Sugar and organic acids released from the pine roots

The results in Fig. 1b showed that the addition of Al had a positive effect on the secretion of glucose. In contrast, the concentrations of fructose and aldose were very low during the entire experimental assay. The Al-stimulated release of glucose can be explained by the fact that Al affects adaptive reactions relating to carbon metabolism. Root-released glucose may be related to selective permeability of the plasma membrane, it can maintain cell osmotic potential and alleviate the physiological, metabolic imbalance caused by Al stress (Yao et al. 2020). Yang et al. (2020) also reported the flow of glucose, fructose, total soluble sugars to organic acids metabolism in Al-treated roots. Acidic rhizosphere contributed to the release of glucose, fructose and total soluble sugars, thus alleviating Al-toxicity. The study by Yan et al. (2019) further confirmed that Al stress could stimulate the glucose formation in plants, similar to our results; besides, lower sucrose contents were found in Al-treated roots. The reason may be that the induction of soluble sugar improves the osmotic regulation ability of cells, enhances the water holding capacity of cells and reduces the damage caused by Al stress (Piñeros et al. 2005). Moreover, Al stress plants decompose starch and sucrose to form a large amount of glucose as energy matrix, which provides sufficient energy for plant growth, thereby improving the ability of plants to resist Al toxicity (Martins et al. 2013).

The root-released organic acids are another observed physiological change in response to added Al. The results in Fig. 1c showed that Al quantitatively stimulated the efflux of oxalic acid from excised root apices of the pine seedlings. Clearly, organic acids have been directly implicated in a number of rhizosphere processes such as Al-detoxification



and nutrient solubilization by plant roots. The Al-induced secretion of malic acid has been reported as an Al-tolerance mechanism by Yao et al. (2020). Their direct role in these rhizosphere processes, however, has been difficult to establish due to the many interdependent factors influencing the release of organic acids. These factors include solid phase sorption/desorption reactions, metal ion complexation reactions, leaching and microbial degradation (Ramesh et al. 2018). Our results confirm that in Masson pine, as in other herb, the release of an Al chelator such as oxalic acid might partly alleviate the toxic effects of Al on tree species.

### Amino acids and secondary metabolites released from the pine roots

Rhizosphere Al regulated amino acid secretion, making several original amino acids disappear and some new amino acids appear (see Fig. 2). Compared to control treatment (without Al), there was obvious difference in the metabolic patterns of amino acids in Al-stressed roots. Interestingly, Al exposure caused severe physiological metabolize in Masson pine and triggered much a small stimulation in certain amino acids. 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 finding of Yan et al. (2019) further confirmed that that a large number of genes are involved in the transport and metabolism of amino acids in plant roots. Al stress may be alleviated by regulating amino acids biosynthesis and metabolism. The metabolic change of amino acids can alleviate Al phytotoxicity by maintaining plasma membrane stability.

The composition of secondary metabolites released from the roots was different after exposure to different Al treatments. As shown in Fig. 3, some original secondary metabolites could be detected in blank assay, however, under condition of Al treatments, there were some new secondary metabolites to be later found. These new secondary metabolites are an interesting response to Al stress, having developed strategies to avoid or tolerate Al-induced effects. More information has to be included about transporters and ion channels, which could give hints on the release of secondary metabolites from plant roots. Secondary metabolites are endogenous signaling molecules that modulate several biotic and abiotic stresses, including Al resistance in plants (Lan et al. 2016). Meanwhile, a significant accumulation of toxic secondary metabolites, such as phenolic acids, that could become injurious in the low-pH rhizosphere and further reduce the pH values of soil (Ma et al. 2016). In addition, a large number of genes are involved in the transport and metabolism of secondary metabolites in plant roots to alleviate Al stress (Yan et al. 2019). Al-induced transporters are capable of transporting various molecular substrates,

including secondary metabolites (Riaz et al., 2018; Yang et al., 2019). However, there is no more comprehensive theoretical study on the secretion of secondary metabolites. Little is known about the ability to transport secondary metabolites.

### Endogenous hormones in the pine roots

The rooting response of ABA, GA<sub>3</sub>, IAA, and ZR to increased Al levels was presented in Fig. 1d. In this study, we obtained a relationship between these hormones and high-Al tolerance. The significant variation of ABA and GA<sub>3</sub> in Al-treated roots seems to be associated with Al response, but the opposite responses to Al were observed for ABA and GA<sub>3</sub>. However, how the dose–response relationship is associated with Al-inhibition of root growth may be governed by complicated metabolic mechanism involved in Al stress signaling which still remain unclear. Preliminary studies indicated that these endogenous hormones were regulate root responses to Al, which controlled the physiological processes and were of particular significance given their role in the protective responses of plants against Al stress (Haruta and Constabel 2003). Similar to this study, Zhang et al. (2014) documented the changes in endogenous level of IAA in root tips after Al exposure. Zhang et al. (2018) also described that ABA, as a stress hormone, played a central role in adaptive responses to environmental stress. Whether Al-inhibition of root elongation is related to expression of genes involved in endogenous hormone metabolism also need to be answered clearly. This possibility of the interpretation will be the focus of future work in our research.

### 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  $\mu\text{g g}^{-1}\text{dw}$ . Most trees contain no more than 0.2  $\mu\text{g g}^{-1}\text{dw}$  of Al (Zhang et al. 2014). The Al<sup>3+</sup> 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 resulted in a reduced root system (Ren et al. 2022). Long-term exposure to Al, such as 100 days, would lead to nutrient deficiencies, mainly of P, K, Ca and Mg, and then inhibition of root growth generally (Vitarello et al. 2005; Zhang et al. 2019a, b). Zhang et al. (2014) observed that root growth was immune to lower level additions of Al.

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

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

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

The pine seedlings emitted different physiological signal in response to Al exposure in pine rhizosphere (Yan et al. 2019). Many compounds released from the pine roots (such as electrolyte,  $\text{H}^+$ , sugar, organic acids, amino acids, endogenous hormones, enzymes and other secondary metabolites) reacted or balanced with external Al ions to alleviate Al toxicity. The composition of root-released compounds is related to Al-resistance. The observed Al-induced changes in the root-released compounds were a response to Al that could contribute to alleviate Al toxicity.

Finally, Al stimulates the efflux of oxalic acid by activation of anions channels (Yang et al. 2020). Activation of anion efflux channels facilitating the efflux of oxalic acid seems responsible for Al resistance. The Al-induced organic acid anions were transported through the specific anion channel across the plasma membrane into plant rhizosphere, which made the plasma membrane positively charged and rejects external  $\text{Al}^{3+}$  (Lan et al. 2016) as well as  $\text{H}^+$  (Zhang et al. 2019a, b). The oxalic acid stained inside root tip may react with smaller amounts of Al. These complexes are not toxic to plant cells (Riaz et al. 2018). Outside Masson pine roots, the secreted oxalic acid reacted or balanced with external Al to obtain Al tolerance (Wang et al. 2020). The stable Al-organic acid complexes do not cross rooting plasma membrane and cannot be accumulated the pine roots, which can contribute to alleviate Al toxicity (Gomez-Zepeda et al. 2021).

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 (Tan et al. 2005). 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, this study gives important evidence on the root exudation of organic compounds from Masson pine treated with Al in hydroponics. Here, we explored 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, glucose, fructose + aldose, amino acids, endogenous hormones and secondary metabolites), Al uptake and accumulation, root growth of Masson pine cultivar. The pine root defense system gradually showed 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, regulatory processes and metabolic mechanism, which may play an important role in regulating Al-resistance of *Pinus nassoniana*, as for how they modulate these rhizospheric processes is in progress. These findings contribute to understanding of root physiological responses to Al toxicity of forest trees. Additionally, exposure to Al also influences  $\text{H}^+$ -adenosine triphosphatase ( $\text{H}^+$ -ATPase) activity in Masson pine (Minorsky 2019). The biochemistry of Al and the  $\text{H}^+$ -ATPase mechanisms by which it affects Al tolerance merits future investigation.

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## Declarations

**Conflict of interest** No potential conflict of interest was reported by the authors.

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