Plant Soil (2019) 445:259–271
https://doi.org/10.1007/s11104-019-04296-3

REGULAR ARTICLE

Effects of different inhibitors such as malonic acid, Na_3PO_4 and $HgCl_2$ on uptake of different forms of antimony in rice plant

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Received: 22 May 2019 / Accepted: 2 September 2019 / Published online: 11 October 2019
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Abstract
Background and aims Antimony is an analogue of arsenic (As), but its uptake mechanisms are not as well understood as As. Antimonite [Sb(III)] probably enters into plant roots via aquaporins but antimonate [Sb(V)] not through the phosphate [P(V)] uptake system as with arsenate [As(V)]. However, previous studies observed a dose–dependent interaction between As(V) and P(V) in some plants. This study was conducted mainly to identify that 1) whether the uptake of Sb(III) by plants will be via aquaporin channels; 2) whether the interaction effects between Sb(V) and P(V) might be dose–dependent; 3) whether the uptake of Sb(III) or Sb(V) is at the cost of energy.

Methods Two hydroponic culture systems were set up using a rice plant (YeXiangYou No.3) to investigate the effects of different chemicals on the uptake of Sb in the rice plants subjected to Sb(III) and Sb(V). These chemicals included malonic acid ($C_3H_4O_4$), Na_3PO_4 [P(V)] and $HgCl_2$.

Results Sb was mainly sequestrated in the roots of the rice plants, suggesting a low transport capacity of Sb from roots to shoots. The plants took up Sb more easily under Sb(III) exposure than under Sb(V) exposure. 10 mg L^{-1} Sb(III) increased the Sb concentration in the bleeding sap rather than the weight of the bleeding sap; but the situation reversed when rice plants were exposed to Sb(V), suggesting different transport mechanisms of Sb from roots to shoots between Sb(III) and Sb(V). The addition of $C_3H_4O_4$ generally reduced the Sb concentrations in the shoots and roots subjected to Sb(V), suggesting the uptake of Sb(V) to be energy dependent. The addition of Na_3PO_4 also significantly reduced the concentrations of Sb in the shoots and roots when plants were exposed to Sb(V). Interestingly, the addition of $HgCl_2$ significantly reduced the concentrations of Sb in the shoots and roots when rice plants were exposed to both Sb(III) or Sb(V), possibly implying that uptake of Sb(III) might be via aquaporins and Cl^- played a role in affecting the uptake of Sb(V).

Conclusions The results of this study suggested that uptake of Sb(III) is via aquaporins, and Cl^- as well as PO_4^{3-} may compete with Sb(V) for uptake pathway.

Responsible Editor: Juan Barcelo.

Electronic supplementary material The online version of this article (https://doi.org/10.1007/s11104-019-04296-3) contains supplementary material, which is available to authorized users.

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Keywords Bleeding sap · Antimonite · Antimonate · Inhibitors · Uptake mechanisms

Introduction

Antimony (Sb) is not an essential element for humans and animals. The mechanisms responsible for antimonite [Sb(III)] and antimonate [Sb(V)] uptake in living organisms are not as well understood as arsenic (As, an analogue of Sb) at this time. Several reports have documented that some aquaporins are responsible for the uptake of Sb(III) in microorganisms. For example, Sanders et al. (1997) reported that the GlpF (a glycerol facilitator) was responsible for uptake of Sb(III) in *Escherichia coli*. Meng et al. (2004) also found that the uptake of Sb(III) was reduced when the gene encoding GlpF was deleted, which suggested that the GlpF protein was involved in the uptake of Sb(III) by cells of *Escherichia coli*. Other reports have shown that the Fsp1 gene (encodes a glycerol transporter that is homologous to the *Escherichia coli* glycerol facilitator GlpF) in *Saccharomyces cerevisiae* (Wysocki et al. 2001). The LmAQP1 protein in *Leishmania major* and LtAQP1 proten in *Leishmania tarentolae* (these *Leishmania* proteins have the conserved signature motifs of aquaglyceroporins) were responsible for the uptake of Sb(III) (Gourbal et al. 2004).

Currently, no direct evidence has been obtained to identify the uptake strategy for different forms of Sb in plants. However, rice plants showed a much higher efficiency in taking up Sb(III) and a greater affinity of rice roots for Sb(III) than Sb(V) (Ren et al. 2014). Sb enters plant cells via the uptake systems for some essential elements or biological molecules (Tschan et al. 2009a). The Sb(V) uptake in plants is different from Sb(III) because arsenite [As(III)] inhibited the Sb(III) uptake but did not affect the uptake of Sb(V)(Brochu et al. 2003).

In terms of the Sb(V) uptake strategy in plants, it is speculated that Sb(V), unlike like As(V), is not taken up by the phosphate P(V) uptake system because of the different spatial structure of Sb(V) and P(V) (Tschan et al. 2008). AsO_4^{3-} [resembling P(V)] displays a tetrahedral structure whereas $Sb(OH)_6^-$ is present as an octahedral structure (Tschan et al. 2008). As an analogue of As(V), P(V) addition often shows an antagonism on As(V) uptake. However, in some cases there were no antagonistic effects reported on uptake between As(V) and P(V). For example, Tu and Ma (2003) found that low levels of As(V) stimulated the uptake of phosphorus in *Pteris vittata*; Chen et al. (2002) observed that < 400 mg kg^{-1} P(V) did not affect but > 400 mg kg^{-1} P(V) stimulated the uptake of As in *Pteris vittata*. Hence, the interaction between P(V) and As(V) might be dose−dependent or plant species−dependent. The interactions between As(V) and Sb(V) also showed a dose−dependent manner just like the interaction of As(V) and P(V). For example, addition of As(V) stimulated the uptake of Sb in some fern plants subjected to Sb(V), such as *Pteris cretica* L. (Feng et al. 2011) and *Pteris vittata* (Müller et al. 2013; Wan et al. 2016). However, Feng et al. (2011) found that As uptake was suppressed when Sb was co − present at a high level (20 mg L^{-1}), suggesting a dose−dependent interaction between As(V) and Sb(V). Unlike the general inhibitory effects of P(V) on As(V) uptake, the addition of P(V) at 3 mg L^{-1} was found to show no significant effects on Sb(V) uptake in *Helianthus annuus* (Tschan et al. 2008). Whereas, Tisarum et al. (2015) found that 6.5 mM P(V) decreased Sb(V) uptake in P − sufficient *Pteris vittata*.

Meharg and Jardine (2003) demonstrated that As(III) inhibited the uptake of Sb(III) in rice plants (*Oryza sativa*), suggesting the same uptake pathway for both Sb(III) and As(III). It is well established that plants take up As(III) via aquaporins (Meharg and Jardine 2003). Aquaporins in most plants are sensitive to $HgCl_2$ which has been widely used as an inhibitor of aquaporin channels (Wang et al. 2010) by binding with specific cysteine residues of aquaporins (sulfur−bound mercury) and thus impeding the passage of water or solutes (Kaldenhoff and Eckert 1999). Just as stated above, Sb(III) uptake in microorganisms was found to be via aquaporins, and probably also in plants, which needs to be verified.

The citric acid cycle (TCA cycle) is a common pathway for the complete oxidation and breakdown of sugars, proteins and fats, and it is also a hub of energy metabolism (Sweetlove et al. 2010). Malonic acid ($C_3H_4O_4$) can inhibit the activity of succinodehydrogenase (SDH) which is an important enzyme of the TCA cycle (Bentley 1952), thus disturb the TCA cycle and ultimately reduce the production of ATP (adenosine triphosphate). Currently, the information about whether the uptake of Sb(III) or Sb(V) is at the cost of energy is scarce.

Therefore, this study was conducted using a rice plant (YeXiangYou No.3) in two hydroponic experiments to investigate: 1) the differences in uptake of

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Sb(III) and Sb(V), and the differences in their transports from roots to shoots via monitoring xylem sap; 2) the effects of different inhibitors on the uptake of Sb(III) and Sb(V). Furthermore, Wang et al. (2010) found that As uptake by *P. vittata* was enhanced by Hg, indicating that As(III) uptake by *P. vittata* may not be via Hg − sensitive aquaporin channels or the 10 μM concentration of Hg was not high enough to cause As inhibition in *P. vittata*. Therefore, in this study we selected relatively high levels of $HgCl_2$ to illustrate the interactions between Hg and Sb. In the study of Tschan et al. (2008), a series doses of Sb(V) (0, 3, 6, 12, 18, and 24 mg L^{-1}) was added to the nutrient solution, but only one level of P(V) at 3 mg L^{-1} was used. In this study, we chose relatively high levels of P(V) to investigate the interactions between Sb(V) and P(V).

Materials and methods

Experimental designs and treatment process

Indica type rice (YeXiangYou No.3) was selected for this study. Full seeds of this rice plant were sterilized in 2% H_2O_2 for 20 min. Then the seeds were rinsed several times first with tap water then with de − ionized water, and thereafter sown in a mixed medium of perlite and vermiculite (V: V = 1: 1). When the seedlings had two true leaves, they were transplanted into a 12 L plastic pot containing a half−strength Espino nutrition solution to acclimate for 3 weeks. Thereafter, seedlings with similar size were selected, carefully rinsed with tap water and de − ionized water, and then transferred into a plastic pot containing a 1 L half−strength Espino nutrition (pH: 6.0) solution and different treatment concentrations of Sb(III) and Sb(V). The 50% strength Espino nutrition comprised of 2.5 mM $Ca(NO_3)_2$•$4H_2O$, 2.5 mM KNO_3, 1 mM NH_4NO_3, 0.25 mM K_2SO_4, 1 mM $MgSO_4$•7 H_2O, 0.5 mM KH_2PO_4, 80 μM EDTA−Fe, 20 μM H_3BO_3, 4.5 μM $MnCl_2$•$4H_2O$, 0.3 μM $ZnSO_4$•$7H_2O$, 0.16 μM $CuSO_4$•$5H_2O$, 0.16 μM $(NH_4)_6Mo_7O_{24}\cdot7H_2O$. Sb(III) and Sb(V) were added in the forms of potassium antimonyltartrate [$K_2Sb_2(C_4H_2O_6)_2\cdot3H_2O$] and $KSb(OH)_6$, respectively. The seedlings were cultured in a glass greenhouse and the growth conditions of the seedlings were as follows: a 25–30 °C temperature range and a 35–50 RH% relative humidity and natural light. There were two experiments in this study and the experimental design was as follows. In both experiments, the nutrient solutions were not aerated.

Experiment I: This experiment was designed to investigate the capability of rice plants to transfer Sb via the xylem. There were in total five treatment levels including the control (CK, 0 mg L^{-1}), 10 mg L^{-1} Sb(III) [$Sb(III)_{10}$], 20 mg L^{-1} Sb(III) [$Sb(III)_{20}$], 10 mg L^{-1} Sb(V) [$Sb(V)_{10}$], and 20 mg L^{-1} Sb(V) [$Sb(V)_{20}$]. Each treatment was repeated six times. After the addition of different speciation of Sb to the nutrient solution for one day, the aboveground of three seedlings were cut at 2 to 3 cm above the roots. The surface of the cross section was rinsed with de − ionized water for several times and blotted up by filter papers. Then the cross section was surrounded by weighed degreasing cotton (it was wrapped by plastic wrap). Fifteen hours later, the degreasing cotton was weighed again and then digested for determination of elemental concentrations. The remained seedlings of three replications were left to grow 2 weeks in order to determine the total concentration of Sb. The solution was replaced every three days. The solution pH was adjusted to 6.0 with 0.1 M NaOH and HNO_3. The seedlings were washed with tap water and then with de − ionized water. Thereafter, the water adhering to the surface of the seedlings was blotted up by filter papers, and then the plants were separated into shoots and roots. The shoots and roots were oven dried at 70 °C to a constant weight. The oven−dried samples were ground and digested for the measurement of elemental concentrations.

Experiment II: This experiment was carried out to study the effects of different inhibitors on the uptake of Sb in rice plants in a 1 L half−strength Espino nutrition (pH: 6.0) solution. Two kinds of Sb speciation [Sb(III) and Sb(V)] and three kinds of inhibitors, including malonic acid, Na_3PO_4, and $HgCl_2$, were selected for this experiment. Antimony was supplied at two concentrations of 10 and 20 mg L^{-1}, and the inhibitors were added at two concentrations of 5 and 10 mg L^{-1}. Hence, there were in total 29 treatments including the control (without Sb and inhibitors) as shown in Table 1 (Supplemental Tables). Each treatment was performed in triplicate. The solution pH was adjusted to 6.0 with 0.1 M NaOH and HNO_3. Three days

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after the addition of Sb and different inhibitors, the seedlings were harvested. The harvesting and subsequent processes of seedlings, such as oven−drying, were similar to Experiment **I**.

Digestion of the samples

The samples of the shoots, roots and degreasing cotton were digested using an ED54 DigiBlock digestion system (Lab Tech, Inc., Hopkinton, MA, USA) according to the method described in Liao et al. (2016). The digestion process was as follows: approximate 0.2 g plant samples was weighed into digestion tubes, and then 10 mL of concentrated HNO_3 was added. The above mixture was incubated overnight. After that the mixture was heated at 80 °C for 1.5 h, then at 120 °C for 1.5 h and finally at 150 °C for 3 h. Thereafter, the temperature was adjusted to 170 °C until the volume in the tubes was approximately 1 mL. After that the 1 mL solution was cooled, filtered into a 50 mL volumetric flask, and diluted to a constant volume. Elemental concentrations were determined by inductively coupled plasma mass spectrometry (iCAP Qc ICP−MS, Thermo Fisher, USA). Standard reference material (bush leaves, GBW07603, GSV−2, Center for Standard Reference of China) was used to ensure the accuracy of the analysis. The values of Sb concentration in this standard reference material was determined to be 0.102 ± 0.018 mg kg^{-1} (certified values is 0.095 ± 0.014 mg kg^{-1}).

Data analysis

Univariate variance analysis plus multiple comparisons (Tukey's test) were used to compare significant differences between different treatments ($P \leq 0.05$). All results were the means of three replications ($n = 3$). SPSS18.0 statistical software was used to analyze the data, and a mapping software (Origin 8.0) was employed to draw the charts.

Results

Uptake of Sb and its translocation via xylem

As compared to the control (CK), the concentrations of Sb in the shoots and roots were significantly elevated with increasing Sb exposure concentrations (Fig. 1). The Sb concentration in the roots was much higher than in the shoots regardless of the Sb speciation. Furthermore, under the same treatment concentration, the Sb concentration in the same tissue of rice plants exposed to Sb(III) was significantly higher than those exposed to Sb(V). The highest Sb concentration was observed in the roots of rice plants subjected to 20 mg L^{-1} Sb(III) reaching up to 882 mg kg^{-1}; however, under the 20 mg L^{-1} Sb(V) treatment, the Sb concentrations in the shoots and roots were only 12.8 and 367 mg kg^{-1} (data not shown), respectively.

Sb(III) did not significantly affect the weight of bleeding sap when compared to the control, despite of that 10 mg L^{-1} Sb(III) increased the weight of bleeding sap up to 32.35% (Fig. 2a). The weight of the bleeding sap was significantly enhanced by Sb(V) exposure being up to 70.59 g at the 10 mg L^{-1} treatment level and two times higher than the control at the 20 mg L^{-1} treatment level. Interestingly, the concentration of Sb in the bleeding sap was significantly enhanced at the 10 mg L^{-1} Sb(III) treatment level, but other treatments of Sb(III) or Sb(V) did not significantly influence the Sb concentration in the bleeding sap (Fig. 2b).

Effects of different inhibitors on Sb uptake in rice plant

When different levels of Sb(III) or Sb(V) were present in the solution, the addition of 5 or 10 mg L^{-1} $C_3H_3O_4$ significantly reduced the Sb concentrations in the shoots and roots compared to individual single Sb(III) or Sb(V) treatments except root Sb concentration in the 10 mg L^{-1} $C_3H_3O_4$ plus 10 mg L^{-1} Sb(III) treatment (Fig. 3a–d).

In the Sb(III) treatment, phosphate only reduced Sb uptake in the roots of the Sb(III) 20 mg L^{-1} treatment (Fig. 4c). In the shoots, Sb concentrations were also reduced in the Sb(III) 20 mg L^{-1} treatment by both concentrations of phosphate but were only reduced by the 10 mg L^{-1} phosphate concentration in the Sb(III) 10 mg L^{-1} treatment (Fig. 4a). When the plants were subjected to different levels of Sb(V), the addition of phosphate significantly reduced the Sb concentrations in both shoots and roots (Fig. 4b, d).

When plants were exposed to Sb(III), the addition of 10 mg L^{-1} $HgCl_2$ only significantly reduced the root Sb concentration at 20 mg L^{-1} Sb(III) but

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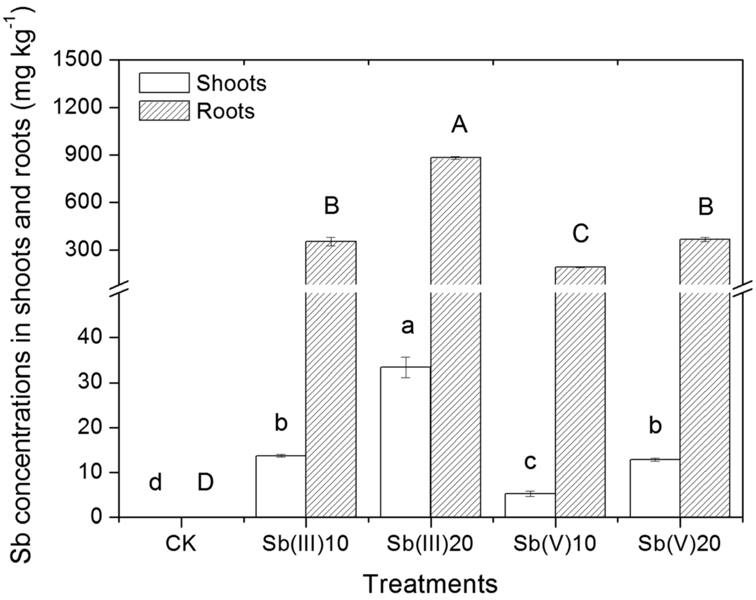

Fig. 1 Concentrations of Sb in the shoots and roots of rice plants subjected to different forms of Sb. Each treatment was replicated three times. Bars are mean and standard error for the mean of three replications. One-way ANOVA and Tukey' multiple range tests were used to compare the significant differences between different treatments. The different lowercase letters and capital letters above bars indicate significant differences between treatments in shoots and roots, respectively

significantly reduced the shoot Sb concentration in both Sb(III) treatments (Fig. 5a, c). Interestingly, under the 10 mg L^{-1} Sb(V) treatment concentration, the addition of 5 mg L^{-1} $HgCl_2$ significantly enhanced but 10 mg L^{-1} $HgCl_2$ did not significantly affect the concentration of Sb in the shoots (Fig. **5b**). However, in the presence of 20 mg L^{-1} Sb(V) in the solution, the addition of 10 mg L^{-1} $HgCl_2$ significantly reduced the concentration of Sb in the shoots (Fig. **5b**). With the different levels of Sb(V) in the solution, the addition of $HgCl_2$ significantly reduced the concentration of Sb in the roots (Fig. 5d).

Discussion

Rice plants absorbed Sb(III) more easily than Sb(V) and mainly sequestered them in the roots

This study was mainly conducted to investigate the effects of different inhibitors on the uptake of different speciation of Sb, through which to explore the potential uptake mechanisms of them in rice plants. The results showed that the concentrations of Sb in different tissues of the rice plants were elevated with increasing Sb treatment concentrations in the solution. The majority

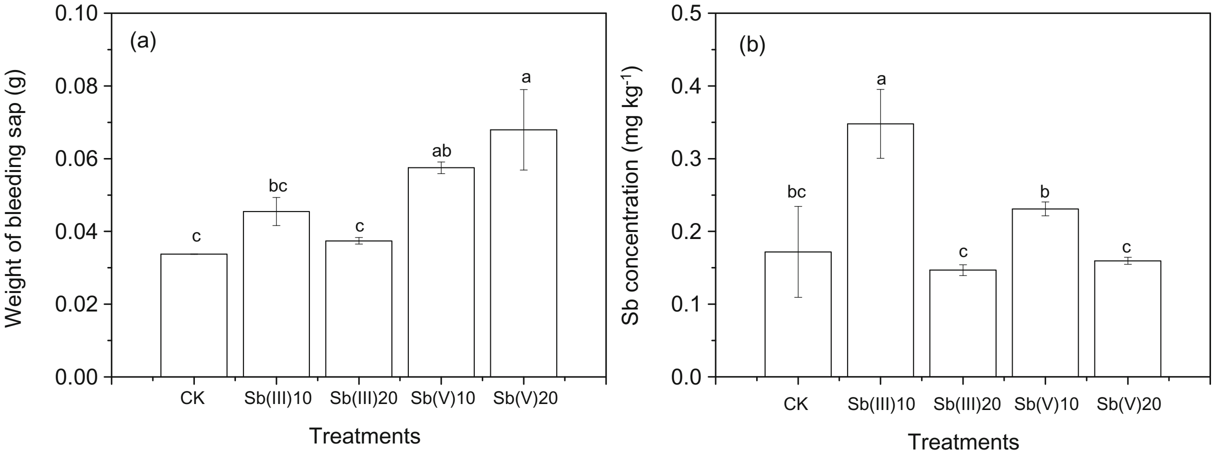

Fig. 2 Bleeding sap weight (**a**) and Sb concentration in the bleeding sap (**b**) when plants subjected to different forms of Sb. Each treatment was replicated three times. Bars are mean and standard error for the mean of three replications. One-way ANOVA and Tukey' multiple range tests were used to compare the significant differences between different treatments. The different lowercase letters above bars indicate significant differences between treatments

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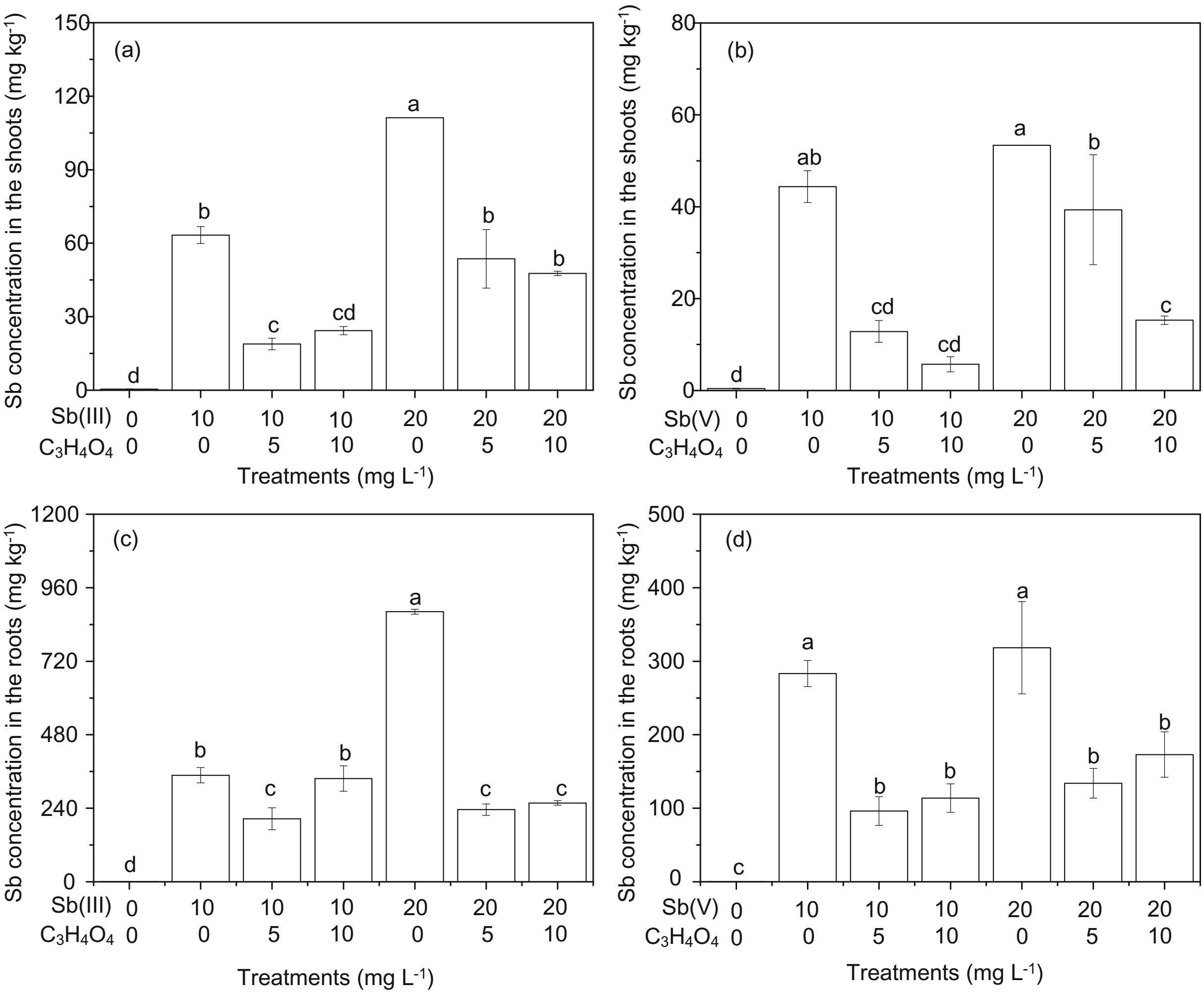

Fig. 3 Effects of malonic acid ($C_3H_4O_4$) on Sb concentrations in the shoots and roots of rice plants subjected to different forms of Sb. Each treatment was replicated three times. Bars are mean and standard error for the mean of three replications. One-way ANOVA and Tukey' multiple range tests were used to compare the significant differences between different treatments. The different lowercase letters above bars indicate significant differences between treatments

of absorbed Sb was concentrated in the roots with the highest Sb concentration being 33.4 mg kg^{-1} and 882 mg kg^{-1} in the shoots and roots, respectively, showing a strong ability to accumulate Sb. However, in this study, we did not remove root iron plaques, and thus not all Sb detected in roots may be really absorbed into the plants. Many studies have shown that plants can accumulate large amounts of Sb (Hozhina et al. 2001; Pratas et al. 2005), such as macrofungi (mushrooms) (Borovička et al. 2006), wheat (*Triticum aestivum* L. Sella), barley (*Hordeum vulgare* L. Ascona), rye (*Secale cereale* L. Palazzo) and sunflower (*Helianthus annuus* L. Iregi) (Ji et al. 2018). The rice plant sequestered the absorbed Sb mainly in the roots, which was consistent with the results obtained in the study of Feng et al. (2013) and the results in other plants in the study of Hozhina et al. (2001).

The rice plants took up Sb more easily as Sb(III) than as Sb(V) (Fig. 1). Under a 5 mg L^{-1} treatment concentration, the root Sb concentration under Sb(III) exposure was 3.53 times higher than under Sb(V) exposure. Similar results were also reported by Ren et al. (2014) and Tisarum et al. (2015). However, Shtangeeva et al. (2012) pointed out that the Sb concentrations in the roots and leaves of rye seedlings exposed to Sb(V) were higher than in those exposed to Sb(III); but, for wheat the situation was reversed. The above results suggest the

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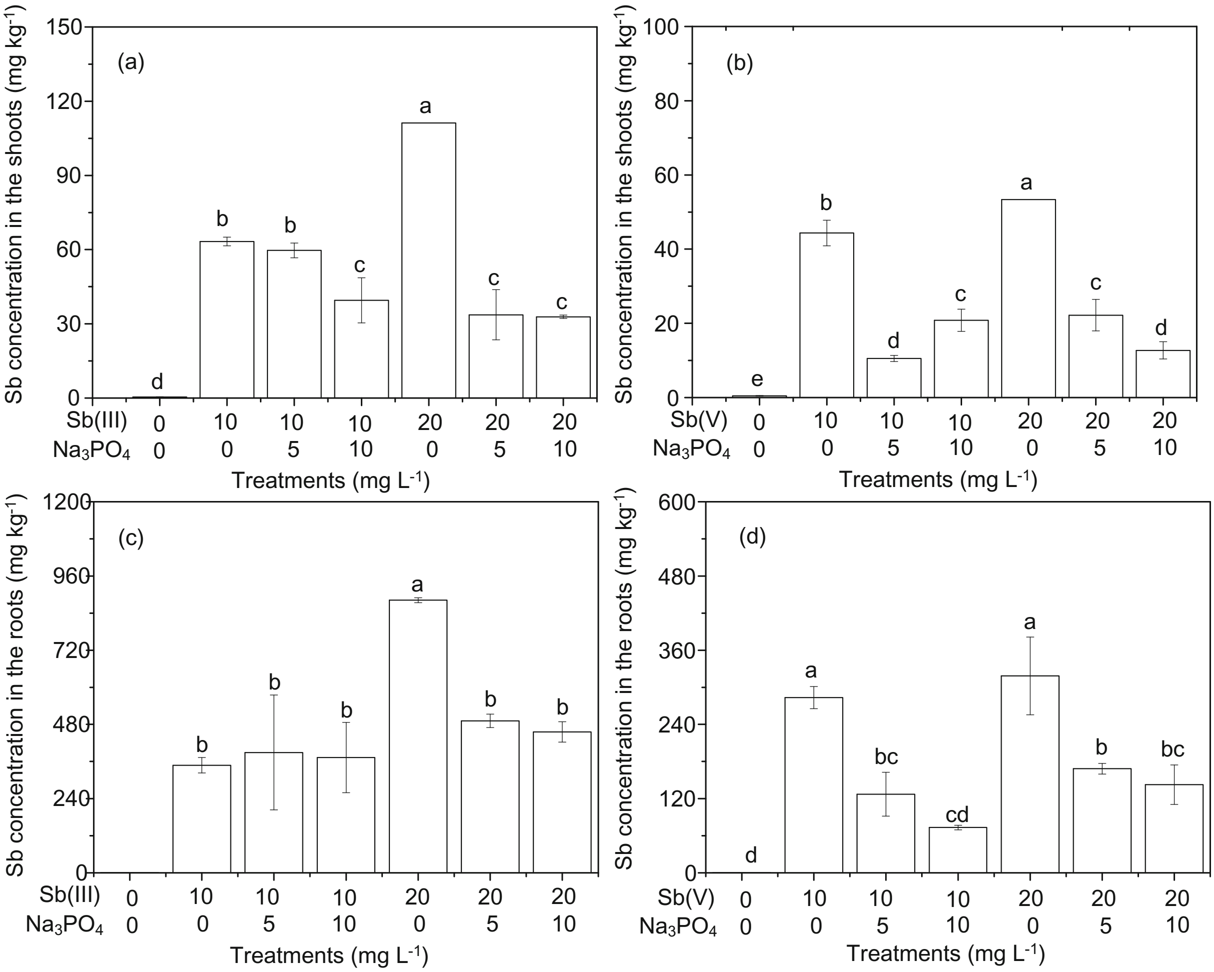

Fig. 4 Effects of Na_3PO_4 on Sb concentrations in the shoots and roots of rice plants subjected to different forms of Sb. Each treatment was replicated three times. Bars are mean and standard error for the mean of three replications. One-way ANOVA and Tukey' multiple range tests were used to compare the significant differences between different treatments. The different lowercase letters above bars indicate significant differences between treatments

capacity of absorbing different speciation of Sb is plant species−dependent.

In this study, we did not monitor the Sb speciation in the solution. However, Sb(V) is reported to be stable in the solution even under reduced condition (Okkenhaug et al. 2012). Another report also observed a relatively stable status for Sb(V) speciation in a hydroponic solution after 7d exposure of Sb(V) (Cai et al. 2016). Sb(III) can be oxidized to Sb(V) especially upon high levels of iron (Fe) hydroxides (Ren et al. 2014); however, Leuz and Johnson (2005) showed no significant oxidation of 98 μg L^{-1} Sb(III) with O_2 in 200 d at pH 3.6–9.8. Ji et al. (2018) reported that less than 20% Sb(III) in nutrient solutions was oxidized to Sb(V) after 8d exposure to Sb(III) for most of the plants used in this study, such as rye (*Secale cereale* L. Palazzo), ryegrass (*Lolium perenne* L. Calibra), wheat (*Triticum aestivum* L. Sella), meadow fescue (*Festuca pratensis* Huds. Preval). In the study of Cui et al. (2015), 92.9% of Sb(III) and 97.7% of Sb(V) were stable in 15 mM Sb(III) and Sb(V) treatments, respectively, within 3d exposure. Ren et al. (2014) stated that rice plant was more efficient in taking up Sb(III) than Sb(V), the amount of Sb(V) taken up by rice plant was probably limited in the Sb(III) treatment. Therefore, in this study, a portion of Sb(III) might be oxidized to Sb(V), but the results that the much higher Sb concentrations in the shoots and roots of rice plants under Sb(III) condition than under Sb(V) condition indicated that a large portion of Sb in the solution remained as Sb(III).

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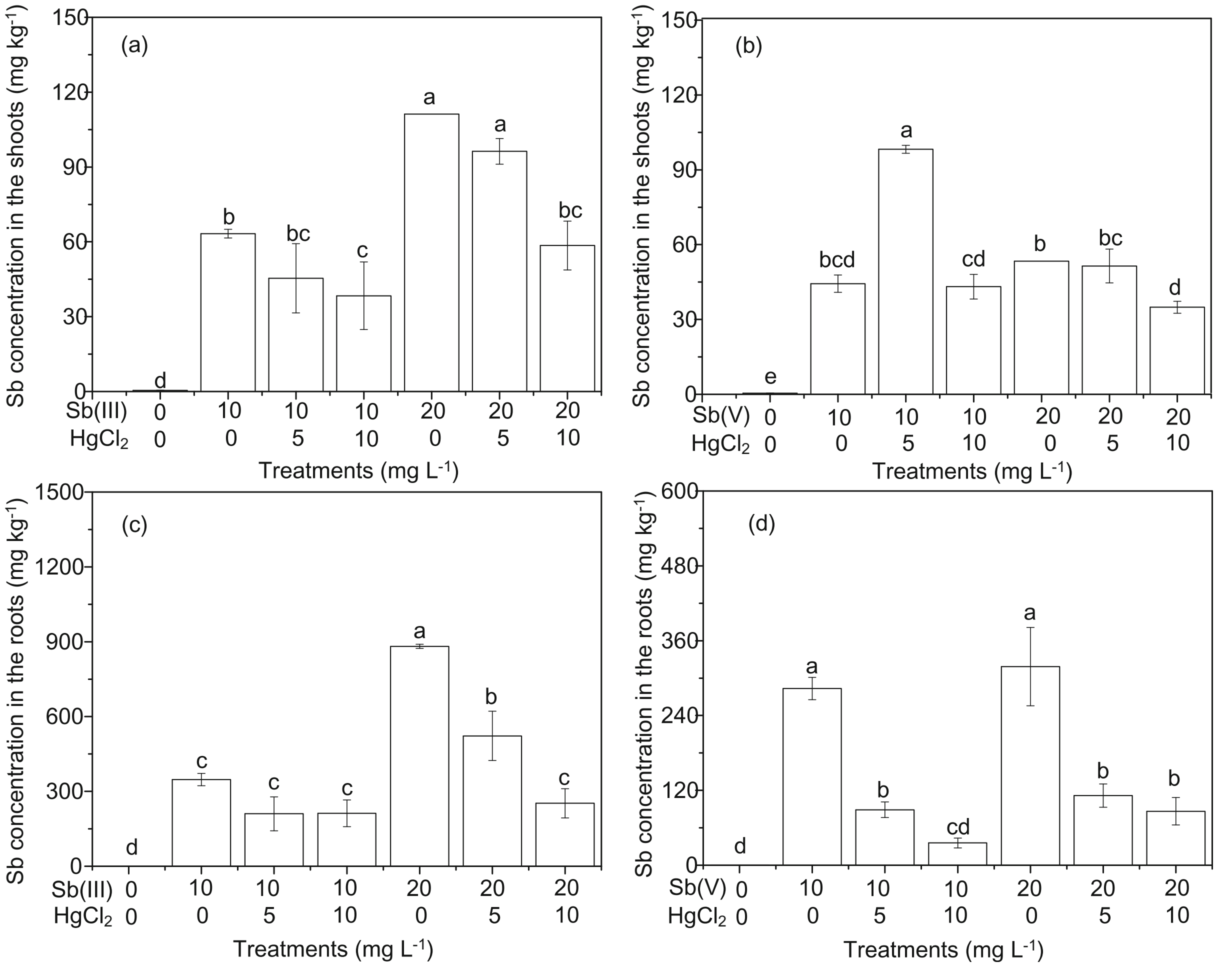

Fig. 5 Effects of $HgCl_2$ on Sb concentrations in the shoots and roots of rice plants subjected to different forms of Sb. Each treatment was replicated three times. Bars are mean and standard error for the mean of three replications. One-way ANOVA and Tukey' multiple range tests were used to compare the significant differences between different treatments. The different lowercase letters above bars indicate significant differences between treatments

Intensity and elemental concentrations of bleeding sap influenced the translocating efficiency of Sb(III) and Sb(V) from roots to shoots in rice plant

The weight of the bleeding sap is closely related to the intensity of root pressure and regarded as a general indicator of root activity (Fu et al. 2019). Xylem sap is often collected to investigate the translocation ability of water, macro− and micronutrients, heavy metals (metalloids), organic anions (Meirer et al. 2007; Ye et al. 2010; Czech et al. 2011; Uroic et al. 2012; Fu et al. 2019). Efficient root uptake and efficient xylem transport are found to be two main factors affecting Cd accumulation (Fu et al. 2019). The results of this study showed that the intensity of bleeding sap was higher under Sb(V) exposure than under Sb(III) exposure (Fig. 2a), suggesting rice plants had stronger root activity and root pressure under Sb(V) exposure. Our previous study pointed out that Sb(V) showed a lower toxicity to rice root growth than Sb(III) (Liu et al. 2019), which is in agreement with the results of a higher bleeding sap weight under Sb(V) exposure than under Sb(III) exposure in this study. In the study of Uroic et al. (2012) and Fu et al. (2019), the addition of As(V) at 100–1000 μM and cadmium (Cd) at 5–10 mg kg^{-1} significantly reduced the bleeding sap intensity, suggesting toxicity of As and Cd to the root growth of related plants. Our results did not show a negative effect of Sb on the intensity of bleeding sap especially under Sb(V) exposure, where the bleeding sap intensity was significantly enhanced

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(Fig. 2a). The above results may indicate a relative high tolerance of this rice plant to Sb or the toxicity of Sb is lower than As and Cd.

Interestingly, exposure to 10 mg L^{-1} Sb(III) significantly enhanced the concentration of Sb in the bleeding sap but other treatments of Sb(III) and Sb(V) did not significantly affect the concentration of Sb in the bleeding sap (Fig. 2b). The actual amount of As transported in the xylem depended on the bleeding sap intensity and the As concentration in xylem sap (Uroic et al. 2012). According to equation in their study (sap flow is multiplied with the total Sb concentration), after 14 h exposure to a level of 10 mg L^{-1}, the absolute amount of Sb under Sb(III) exposure translocated from roots to shoots was 1.19 times higher than that under Sb(V) exposure (data not shown). The above results were in agreement with the higher total Sb concentration in the shoots under Sb(III) exposure than under Sb(V) exposure. This suggested that rice plants translocated Sb from roots to shoots mainly via increasing Sb concentration in the bleeding sap under a relatively low dose of Sb(III) exposure, and Sb(V) as well as Sb(III) displayed different transport patterns in rice plants. However, when exposure to a higher level at 20 mg L^{-1}, the absolute translocated amount of Sb under Sb(III) exposure was 0.51 times higher than under Sb(V) exposure (data not shown). This results cannot be used to explain the still higher shoot and root Sb concentration under Sb(III) exposure than under Sb(V) exposure after 14d exposure of Sb (Fig. 1). In this aspect, we presumed that different growth stage of plants showed a different ability to take up and translocate different speciation of Sb (36 h exposure vs 14d exposure). A time effect for Sb uptake was also observed by Tisarum et al. (2015), in their study the addition of silicic acid ($Na_2SiO_3.5H_2O$), glucose [$CH_2OH(CHOH)_4HCO$] and P(V) impacted Sb(III) uptake during 2 h exposure, but the impact disappeared during 4 h exposure.

Uptake of Sb(V) might be energy−dependent (ATP involved)

In this study, when plants were exposed to Sb(III) or Sb(V), the addition of $C_3H_4O_4$ significantly reduced the uptake of Sb in the shoots and roots (Fig. 3a–d), except that at 10 mg L^{-1} Sb(III) exposure the addition of 10 mg L^{-1} $C_3H_4O_4$ did not significantly affect the root Sb concentration (Fig. 3c). The Sb(V) is stable but a portion of Sb(III) will be probably oxidized to Sb(V) (Ren et al. 2014), so in this study the inhibition of Sb(V) uptake after $C_3H_4O_4$ addition probably indicated that uptake of Sb(V) was energy−dependent (ATP involved). However, we cannot eliminate the possibility that Sb(III) uptake in rice plants may also need energy, which merits further investigation. Similarly, as an analogue of Sb, the uptake of As(III) and As(V) was also reported to need energy (Abedin et al. 2002).

Effects of P(V) on uptake of Sb(V) and Sb(III)

Uptake inhibition of Sb(V) by P(V) might be P − status −dependent

In this study, the addition of these high levels of P(V) significantly reduced the concentrations of Sb both in the shoots and roots (Fig. 4b, d), which was inconsistent with the results of Tschan et al. (2008). In their study, only 3 mg L^{-1} P(V) was added, but in this study we added much higher concentrations of P(V) at 5 and 10 mg L^{-1}. Similar inhibition of P(V) on Sb(V) uptake was also reported by Tisarum et al. (2015). In their study, the re − supply of P(V) (only 2 h) showed no impact on Sb(V) uptake in *Pteris vittata* gametophytes pre − treated with P − starvation for 12 weeks (indicating a low P status in plants); however in P − sufficient *Pteris vittata* gametophytes, the addition of P(V) reduced Sb(V) uptake. Huang et al. (2012b) also observed uptake inhibition of Sb(V) by P(V) in the roots of wild −type rice (*Oryza sativa* L., cv. Oochikara) and its low −silicon mutant (*lsi*1) exposed to 10 μmol L^{-1} Sb(V). The above results implied that the inhibition of Sb(V) by P(V) will occur in the presence of sufficient supplementation of P(V). The uptake inhibition of As by P(V) was also found to be P-status-dependent. For example, in a study of Hu et al. (2005), they found that P(V) addition did not inhibit the As uptake in three rice (*Oryza sativa* L.) cultivars growing in a P-insufficient soil. They suggested that Fe plaque formation was enhanced by P-starvation, thus sequestering more As in Fe plaque and resulting in a vanished inhibition impact of P(V) on As uptake.

The molecular sizes of Sb(V) and P(V) are similar, being 7.36 and 6.24 Å, respectively (Tisarum et al. 2015). Hence, it is possible that they might compete for the same transporter for their uptake. There are two types of P(V) uptake systems (Meharg and Macnair 1990, 1992; Abedin et al. 2002). One has a high affinity (exerted functions under a low P status) and the other

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has a low affinity to P(V) (exerted functions under a sufficient P supplementation) (Abedin et al. 2002). Therefore, it is speculated that the uptake of Sb(V) might be via the P uptake system with a low affinity (high levels of P(V) will compete with Sb(V) uptake). However, the above speculation needs to be further tested by more investigations in the future.

Effect of P(V) on Sb(III) uptake might be dose −dependent

Interestingly, the addition of P(V) significantly reduced the shoot and root Sb concentrations when 20 mg L^{-1} Sb(III) was added (Fig. 4a, c), showing a dose−dependent effect on Sb(III) uptake which seemed to depend on Sb(III) levels in the solution. In this study, this uptake inhibition of Sb(III) by P(V) could not be explained by the same uptake pathway for both Sb(III) and P(V). Because 1) Sb(III) and P(V) have different molecular structures. Reports have shown that in solution at pH 6.0, Sb(V) mainly existed as $[Sb(OH)_6]^-$, while $Sb(OH)_3°$ was the main Sb(III) form (Ren et al. 2014). Phosphate has a much bigger molecular size than Sb(III) (Tisarum et al. 2015). 2) P(V) did not affect the root Sb concentration under 10 mg L^{-1} Sb(III) exposure.

It was also unlikely that P(V) addition stimulated a greater sequestration of Sb(III) in the root Fe plaque, and thus reduced the amounts of Sb entering into the roots. The reason being that in this study, the whole root sample was digested and we did not remove Fe plaque from the root surface. If P(V) addition were to stimulate Sb(III) accumulation in root Fe plaque, the root Sb concentration should be enhanced rather than be constant or decreased (Fig. 4a, c). In the study of Ji et al. (2018), Fe plaque adsorbed a large amount of Sb(III), but which did not affect the root and shoot Sb concentrations in many plants. In addition, Fe plaque often forms on the surface of aquatic plants, but in other non-aquatic plants, similar uptake inhibition of Sb(III) by P(V) was also reported. For example, in the study of Tisarum et al. (2015), P(V) addition significantly reduced the uptake of Sb(III) in a *Pteris vittata* gametophyte pre − treated with P − starvation, but increased that in P − sufficient *Pteris vittata* gametophytes.

One reasonable explanation for uptake inhibition of Sb(III) by P(V) is that the Sb(III) was oxidized to Sb(V), and then P(V) showed an inhibitory effect on Sb(V) uptake. Oxidation of Sb(III) to Sb(V) might be an alternative detoxification method in rye grass (*Lolium perenne* L. Calibra) (Ji et al. 2017). However, in this study the oxidation site of Sb(III) seemed not to be in the solution. Because 1) Leuz and Johnson (2005) found that Sb(III) in solution with O_2 was not oxidized to Sb(V) in 200 d at pH 3.6–9.8; 2) in the study of Tisarum et al. (2015), Sb in the media after 2 h exposure to *Pteris vittata* gametophytes remained the same speciation, but the uptake inhibition of Sb(III) by 6.5 mM P(V) still occurred in P − starvation *Pteris vittata* gametophyte.

Since most of Sb(III) in a slurry solution (pH 6.0) was reported to be oxidized by amorphous Fe and manganese oxyhydroxides (Belzile et al. 2001), the oxidation sites of Sb(III) in this study might be in root Fe plaque rather than in solution. A similar hypothesis was given by Ji et al. (2018), they proposed that Sb(III) was oxidized by dissolved oxygen while being adsorbed on the Fe plaque and then released as Sb(V) into the solution. Fe plaque forms on root surface containing ferric oxides, and adsorption of Sb(III) onto Fe hydroxides may cause the rapid oxidation of Sb(III) to Sb(V) (Ren et al. 2014). Consequently, the significant inhibition of Sb(III) by P(V) in this study may be via the following processes: 1) increasing Sb(III) exposure levels enhanced the formation of Fe plaque (Huang et al. 2012a; Ren et al. 2014; Cai et al. 2016; Ji et al. 2018; Liu et al. 2019); or Fe accumulation in roots [at 20 mg L^{-1} Sb(III), Supplemental Table 2]; 2) Sb(III) in Fe plaque was oxidized to Sb(V) by oxygen or by other oxidizing sources (see below discussion); 3) Fe plaque adsorbed Sb(V) to a lesser extent than Sb(III) (Ren et al. 2014; Ji et al. 2018), so a portion of the Sb(V) from Sb(III) oxidation was released into the solution, resulting the increase in solution Sb(V) concentration.

The above speculation was given mainly based on the more formation of Fe plaque (or excess Fe accumulation in roots) stimulating by increasing Sb(III) exposure concentration. However, P-insufficient can also impact the Fe status in plants. P − insufficiency will lead to a significant increase in Fe concentration in plants (Misson et al. 2005; Zheng et al. 2009), the formation of root Fe plaque and thus the alteration of As(V) uptake in rice plants (Liu et al. 2004), the formation of aerenchyma and thus the adaption to low P condition in maize and common soybeans (Fan et al. 2003), and even Fe toxicity in *Arabidopsis* (Ward et al. 2008). The excess accumulation of Fe can induce the burst of reactive oxidative species (ROS), which act as an oxidation driver for Fe^{2+} in the apoplast, and thus prevent the

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entrance of excess Fe^{2+} into cell inner (Zhang et al. 2011). In the study of Tisarum et al. (2015), they observed that the uptake inhibition of Sb(III) by P(V) only occurred in P − starved *Pteris vittata* gametophyte (for 12 weeks) but not in P − sufficient *Pteris vittata* gametophyte. Although *Pteris vittata* is not an aquatic plant, some non-wetland plants can also form aerenchyma and Fe plaque under aerated hydroponic conditions (Ji et al. 2018). In the study of Tisarum et al. (2015), the hydroponic culture manner and P-starvation for 12 weeks might both facilitate the formation of aerenchyma and Fe accumulation in roots. High Fe uptake may inhibit translocation of Sb(V) with the stream of xylem water (Ji et al. 2018). Therefore, the uptake inhibition of Sb(III) by P(V) in P − starved *Pteris vittata* gametophyte was probably due to oxidation of Sb(III) to Sb(V) by the formed Fe plaque or ROS burst (more likely) resulting from Sb(III) stress and/or excess Fe, and thus had P(V) show an antagonistic effect on Sb(V) uptake. Therefore, more investigations are needed to investigate the correlation between root Fe status, P status, oxidative stress and oxidation process of Sb(III) in plants or on plant root surface.

Uptake of Sb(III) is via aquaporins and Cl^- might affect Sb(V) uptake

In this study, we found that under Sb(III) exposure, the addition of $HgCl_2$ significantly reduced the concentrations of Sb both in the shoots and roots [only at 20 mg L^{-1} Sb(III)] (Fig. 5a, c). The above results suggested that the uptake of Sb(III) might be via aquaporins, because $HgCl_2$ is one inhibitor of aquaporins (Wang et al. 2010) and the uptake of As(III) (Meharg and Jardine 2003) and selenite (Zhang et al. 2010) was reported to be mainly via aquaporins. The inhibition of Sb(III) uptake by $HgCl_2$ may be due to the inhibition of aquaporin activity. The finding of Flynn et al. (2003) that As(III) inhibited the uptake of Sb(III) in rice plant (*Oryza sativa*) can be used to indirectly support our results in this study.

Unexpectedly, the addition of $HgCl_2$ significantly reduced the concentration of Sb in the roots of rice plants subjected to Sb(V) (Fig. 5d). Just as mentioned above, the Sb(V) in the solution is stable. Therefore it is not likely that the reduction of Sb(V) to Sb(III) resulted in the inhibition of Sb uptake by Hg. In the study of Tschan et al. (2009b), they speculated that uptake of Sb(V) into the root symplast would require anion transporters of low selectivity, in which Sb(V) anions could substitute for essential nutrient anions such as Cl^- or NO_3^-. Therefore, the competition between Sb(V) and Cl^- for the uptake pathway might be the reason for the inhibition of Sb(V) uptake by $HgCl_2$.

Conclusions

This study was conducted to investigate the effects of different speciation of Sb on the uptake and transport of Sb. In addition, the effects of different inhibitors including $C_3H_4O_4$, Na_3PO_4 and $HgCl_2$ on the uptake of Sb in rice plants subjected to different speciation of Sb were also studied. The results showed that rice plants could accumulate a large amount of Sb especially under Sb(III) stress in the roots. Uptake of Sb(V) by rice plants used in this study probably needs energy. The uptake of Sb(III) in rice plants might be via aquaporins and Sb(V) appeared to compete with Cl^- for the uptake pathway. In addition, the uptake of Sb(III) and Sb(V) was also significantly inhibited by the addition of P(V).

Acknowledgements This research was supported by the National Science Foundation of China (41473114, 41877497).

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