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REGULAR ARTICLE

A comparison of antimony accumulation and tolerance among *Achillea wilhelmsii*, *Silene vulgaris* and *Thlaspi arvense*

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Abstract
Aims The uptake and tolerance of antimonite [Sb(III)] and antimonate [Sb(V)] were investigated in two populations of *Achillea wilhelmsii*, one from strongly Sb-enriched mine soil, the other from uncontaminated soil, in comparison with non-metallicolous *Silene vulgaris* and *Thlaspi arvense*.
Methods Tolerance was assessed from root elongation and biomass accumulation after exposure to a series of concentrations of Sb(III) or Sb(V) in hydroponics.
Results For all the species Sb(III) was more toxic than Sb(V). *S. vulgaris* was the most Sb(III)-tolerant species, and *A. wilhelmsii* the most Sb(V)-tolerant one. There were no considerable interspecific differences regarding the root and shoot Sb concentrations. Sb(III) and Sb(V) tolerance and accumulation were not different between the metallicolous and the non-metallicolous *A. wilhelmsii* populations. Sb(III) uptake was partly inhibited by silicon. Sb(V) uptake was strongly inhibited by chloride.
Conclusions There is uncorrelated variation among species in Sb(V) and Sb(III) tolerance, showing that plants sequester Sb(V) and Sb(III) in different ways. Sb(V) seems to be taken up via monovalent anion channels, and Sb(III) via silicon transporters, at least in part. The relatively high Sb(V) tolerance in *A. wilhelmsii* seems to be a species-wide property, rather than a product of local adaptation to Sb-enriched soil.

Keywords Antimony · Accumulation · Tolerance · *Achillea wilhelmsii* · *Silene vulgaris* · *Thlaspi arvense*

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Introduction

Antimony (Sb) is commonly used in a broad variety of industrial products (Smichowski 2007). It is a toxic element, and excess intake results in various diseases in humans, including cancer, cardiovascular diseases, respiratory diseases, and liver diseases (WHO 2003). Although Sb, and Sb compounds, are listed as priority contaminants by USEPA and the EU since the early 1970s (EU 1998; USEPA 1979), concerns have been raised only in recent years, because of increasing levels of Sb pollution in the environment. Anthropogenic activities, such as metal (metalloid) mining, smelting and the burning of fossil fuels, have led to the release of a large amount of antimony into the environment, causing serious Sb pollution in some regions of the world.

Antimony is not essential for plants but can be readily absorbed by roots when it occurs in soluble form (Baroni et al. 2000). Accumulation and toxicity of Sb in plants have gained increasing attention in recent years. The phytotoxicity threshold level of Sb in plant tissue has been reported to be in the range of 5–10 mg kg^{-1} (Kabata-Pendias and Mukherjee 2007). However, Eikmann and Kloke (1993) reported that 5 mg kg^{-1} is tolerable for plants.

Previous studies have shown that several plant species can accumulate Sb at high concentrations from Sb-contaminated soil. Foliar Sb concentrations of up to 1100 mg kg^{-1} were measured in plants growing in a soil containing up to 400 mg Sb kg^{-1} dry weight (DW) in the vicinity of a Sb smelter in northeast England (Ainsworth et al. 1990). Another study reported foliar Sb concentrations above 100 mg kg^{-1} in plants growing on a mine tailing with 9000 mg Sb kg^{-1} DW. At the same site, more than 1000 mg Sb kg^{-1} DW was found in the basal leaves of *Achillea ageratum* (Baroni et al. 2000). Other studies reported only low Sb concentrations in plants grown on heavily Sb-contaminated soils. Pratas et al. (2005) reported maximum stem concentrations of less than 5 mg Sb kg^{-1} DW in various tree and herb species growing on a Portuguese mine tailing with an average total Sb concentration of 663 mg kg^{-1}. Deposition of Sb-containing dust particles on leaf surfaces may be one of the reasons for the high plant Sb concentrations on contaminated field sites (Ainsworth et al. 1990), and thus to some extent explain the diversity of results reported in the literature, given that most studies did not discriminate between Sb accumulation through dust deposition and root uptake.

Plants can take up Sb in different forms. In general, inorganic Sb compounds were found to be more toxic than organic ones (Filella et al. 2002b). The two common inorganic forms of antimony present in natural environments are antimonate ($Sb[OH]_6^-$) and antimonite ($Sb[OH]_3$), prevailing in oxidized and reduced soils, respectively (Filella et al. 2002b). Experimental and clinical trials with Sb compounds have shown that the trivalent form is generally more toxic than the pentavalent form (Winship 1987; Gebel 1997; WHO 2006).

The biogeochemistry of Sb is poorly known, in comparison with that of other metals or metalloids. It is generally assumed that its geochemical behavior and toxicity are similar to those of arsenic (As), which also exists in the trivalent and pentavalent forms in the natural environment (Filella et al. 2002a; Wilson et al. 2004; Tighe et al. 2005a, b; Gal et al. 2006). Soluble antimonate speciates as a monovalent anion ($Sb[OH]_6^-$) between pH 2 and pH 10, i.e. across the entire range of pH values found in soils. The existence of a specific mechanism for Sb uptake is not likely, because Sb is not biologically essential (Tschan et al. 2009). As an anion, to be passively taken up into a root cell, antimonate has to overcome an electrical potential difference in the range of −100 to −200 mV, which would require an outer concentration of two to three orders of magnitude higher than the internal one (Reid and Hayes 2003). Thus, at least at low external concentrations, uptake of antimonate into the root symplast would require anion transporters of low selectivity, in which antimonate anions could substitute for essential nutrient anions such as Cl^- or NO_3^- (Tschan et al. 2009). Regarding its translocation to the shoot, an alternative route would be via the apoplastic pathway, as has been suggested for negatively charged metal chelates (Bell et al. 2003; Wenger et al. 2005). Passive uptake of antimonite could theoretically be mediated by aqua-glyceroporins, in particular LSI-type silicon transporters, such as demonstrated for arsenite in rice (Ma et al. 2008).

Achillea wilhelmsii C. Koch (Asteraceae) is a herb widely distributed in different parts of Iran, especially in the central and western parts (Rechinger 1986). It is a facultative metallophyte, growing on non-metalliferous soils, but also on metalliferous soils in the old Sb mining areas of northwest Iran (Moghanlo), where it accumulates Sb at considerable concentrations in its shoot (Jamali Hajiani et al. 2015).

The primary objectives of the present work were to study the tolerance and accumulation of Sb(III) and Sb(V) in metallicolous (M) and non-metallicolous (NM) *Achillea wilhelmsii*, using non-metallicolous *Silene vulgaris* and the non-metallophyte *Thlaspi arvense* as reference species. The second aim was to identify the type of transporters responsible for the uptake of Sb(III) and Sb(V) into plant roots. To this end, we also studied the effects of increasing concentrations of nitrate, silicon and chloride in the nutrient solution on the uptake of Sb(III) and Sb(V).

Materials and methods

Seed collection, plant culture, and experimental design

Seeds of *Achillea wilhelmsii* were collected from plants growing in an old Sb mine in the Moghanlo area in

Northwest of Iran (Jamali Hajiani et al. 2015), and from a nearby non-metalliferous site in the Dandi area. Seeds of *Silene vulgaris* and *Thlaspi arvense* were collected from a roadside near the campus of the Vrije Universiteit, Amsterdam, which was un-contaminated with heavy metals, apart from a slight Pb contamination (H. Schat, unpublished). Seeds were sown on a commercial garden soil (Jongkind BV, nr. 7, Alsmeer, The Netherlands), and after 2 weeks seedlings were transferred to hydroponic culture, in 1-L polyethylene pots (three plants per pot) containing a modified half-strength Hoagland's solution composed of 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 1 mM $NH_4H_2PO_4$, 0.5 mM $MgSO_4$, 20 μM Fe(Na)-EDTA, 1 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.1 μM $CuSO_4$ and 0.1 μM $(NH4)_6Mo_7O_{24}$, in demineralized water, buffered with 2 mM 2-(N-morpholino) ethanesulphonic acid (MES), pH 5.5, adjusted with KOH. Nutrient solutions were renewed weekly. The experiments were done in a growth chamber (20/15 °C day/night; light intensity 200 μE m^{-2} s^{-1}, 14 h day^{-1}; relative humidity 75 %).

In a first experiment, plants were exposed to a range of concentrations (0, 1, 3, 9, 27, 81, 243 μM; 4 pots with 3 plants each per comcentration) of antimonate (Sb[V]), or antimonite (Sb[III]), supplied as potassium hexahydroxoantimonate and potassium antimonyl(III)tartratetrihydrate, respectively. At the start of the experiment and once per week thereafter, the pots were randomized within the climate room. Prior to exposure, roots were stained with active carbon powder to facilitate the measurement of root growth (Schat and Ten Bookum 1992). After 6 days of exposure, the length of the longest unstained root segment was measured. The plants were harvested for analysis after having grown in the test solutions for 3 weeks. Prior to harvest, the roots were desorbed for 10 min with 10 mM $CaCl_2$. Plants were divided into root and shoot fractions and samples were air-dried in an oven at 70 °C for 48 h. Then root and shoot dry weights were determined.

In a second experiment, performed exclusively with metallicolous *A. wilhelmsii*, the time course of the redox status of Sb in the nutrient solution was recorded in pots with 3 plants, which had been grown for 3 weeks in an Sb-free nutrient solution. At the start of the experiment, the nutrient solution was replaced by a fresh one amended with 9 or 27 μM of either Sb(III), or Sb(V) (4 pots per concentration per Sb species). To test for any potential effects of plant roots, the same experiment was performed with pots without plants. Samples (1 mL) from the nutrient solutions were taken each day, during 5 successive days, after which the experiment was terminated. In addition, to check for potential Sb(V) reduction inside the roots and subsequent Sb(III) efflux, 6 plants were exposed to Sb(V) for three weeks (as described above), and then placed with their roots in light-tight 50 mL-tubes (1 plant per tube) filled with Sb-free nutrient solution, after a 10-min root desorption in 10 mM $CaCl_2$. Once per day the nutrient solution volume was adjusted to 50 mL with demineralized water, after which a 1-mL sample was taken for Sb analysis. After 5 days the experiment was terminated.

In a third experiment, designed to get some indication of the nature of the uptake mechanisms of Sb(III) and Sb(V), we tested the effects of nitrate (KNO_3), chloride (NaCl), and silicon ($Na_2Si_3O_7$) supply on Sb(III) and Sb(V) uptake in metallicolous *A. wilhelmsii*. To this purpose, plants (4 pots with 3 plants each per treatment per concentration) were grown for 3 weeks in Sb-free nutrient solution, and then exposed to 81 μM of either Sb(III) or Sb(V), both in the presence of different concentrations of nitrate, chloride, or silicon. In the nitrate treatments K^+ and Ca^{++} in the background solution were supplied as K_2SO_4 (1.5 mM) and $CaSO_4$ (2.0 mM), respectively. After 5 days plants were harvested for Sb analysis (see above). Sb(V) was supplied without and with NaCl (2.5 mM), KNO_3 (1500 μM), or $Na_2Si_3O_7$ (1500 μM). Sb(III) was also supplied with and without $Na_2Si_3O_7$ (15, 150, 1500 μM), NaCl (2.5 mM), or KNO_3 (1500 μM).

Sb measurement

Sb concentrations were determined in roots and shoots (4 replicate samples of 3 pooled plants per concentration). Sb was determined by digesting 50–100 mg of oven-dried plant material in 2 mL of a 1 to 4 (*v/v*) mixture of 37 % (*v/v*) HCl and 65 % (*v/v*) HNO_3 in Teflon cylinders for 16 h at 140 °C, after which the volume was adjusted to 10 mL with demineralized water. To reduce all of the Sb, 2 mL of the diluted digest were mixed with 4 mL of ascorbic acid (5 %) and 4 mL of potassium iodide (5 %). Sb(III) and Sb(V) concentrations in the nutrient solution were measured in 1-mL samples that were 10-fold diluted with 2 mM citric acid and then incubated at room temperature (2 h) to allow Sb(III)-citrate complex formation. Solutions were then passed through Sep-Pak Accellplus QMA cartridges (WAT020545) from Waters (Waters Consortium, Milford MA, USA), which retain Sb(III). The first 5 mL of flow-through solution was discarded before collecting the

solution containing only Sb(V) (Tisarum et al. 2014). Samples were treated as above, and Sb was determined on a flame atomic absorption spectrophotometer (Analist100, Perkin-Elmer), using the EDL (Electrodeless Discharge Lamp) system. Freshly prepared solutions of potassium antimonyl(III)tartratetrihydrate in 0.1 M HNO_3 were used for calibration.

Statistics

The data were analyzed using a model 1 two-way ANOVA after log-transformation of the data. The minimum significant range (MSR) statistic was used for post-hoc comparison of multiple individual means (of the log-transformed data) (Sokal and Rohlf 1981). Variation in tolerance among species was statistically tested through comparing the differences between the means (of the log-transformed data) of the control and those of the 243-μM treatments. The variances of these differences were calculated as the sum of the variances of the control and the 243-μM treatments.

Results

Higher concentrations of Sb(III) caused visual toxicity symptoms in all the plant species. At the 81- and 243-μM exposure levels, stunted growth, and foliar chlorosis and necrosis were apparent in all of the plant species and populations, including the M *A. wilhelmsii* population.

The lowest Sb(III) concentration that significantly inhibited root elongation growth was 27 μM in all the species (Fig. 1, Table 1). However, there was a significant species x treatment interaction ($p < 0.001$). Further statistical comparison of the responses showed that *S. vulgaris* was significantly more Sb(III)-tolerant than *A. wilhelmsii*, which was in turn significantly more Sb(III)-tolerant than *T. arvense* (Table 2). There was no statistically significant population x treatment interaction ($p > 0.05$) between M and NM *A. wilhelmsii*. The lowest Sb(III) concentration that significantly decreased root dry weight was also 27 μM in *S. vulgaris*, as well as in M and NM *A. wilhelmsii*, but was only 3 μM in *T. arvense* (Fig. 2, Table 1). Also for root DW the species x treatment interaction was significant ($p < 0.001$), with Sb(III) tolerance decreasing in the order *S. vulgaris* > *A. wilhelmsii* > *T. arvense*. However, for root DW the responses of *S. vulgaris* and the M and NM *A. wilhelmsii* populations were not significantly different (Table 2). For shoot DW the lowest significantly inhibitory Sb(III) concentrations were, for all the species, three times higher than those for root DW, i.e. 81 μM for *S. vulgaris* and M and NM *A. wilhelmsii*, and 9 μM for *T. arvense*, respectively (Fig. 3, Table 1). Also for shoot DW the species x treatment interaction was significant ($p < 0.001$), again with Sb(III) tolerance decreasing in the order *S. vulgaris* > *A. wilhelmsii* > *T. arvense*. Just like for root DW, *T. arvense* was found to be significantly less Sb(III)-tolerant than *S. vulgaris* and *A. wilhelmsii* (both M and NM), which were not significantly different (Table 2).

Sb(V) caused severe foliar chlorosis and necrosis in *S. vulgaris* and *T. arvense*, though exclusively at the 243-μM exposure level. In contrast, *A. wilhelmsii* remained green and healthy at this exposure level.

The lowest Sb(V) concentration that significantly inhibited root elongation was 27 μM for *S. vulgaris* and *T. arvense*, but 243 μM for *A. wilhelmsii* (Fig. 1, Table 1) The species x treatment interaction was significant ($p < 0.001$). *A. wilhelmsii* was significantly more Sb(V)-tolerant than *S. vulgaris* ($p < 0.001$), which was in turn significantly more Sb(V)-tolerant than *T. arvense* (Table 2). There was no significant population x treatment interaction among N and NM *A. wilhelmsii*. Also when estimated from root and shoot DW, *A. wilhelmsii* was significantly more tolerant than *S. vulgaris*, while *S. vulgaris* was significantly more tolerant than *T. arvense* (Table 2), and there was no significant species x treatment interaction among M and NM *A. wilhelmsii*. The lowest significantly inhibitory Sb(V) concentrations for root DW were 27, 243, and 3 μM for *S. vulgaris*, both M and NM *A. wilhelmsii*, and *T. arvense*, respectively (Fig. 2, Table 1). For shoot DW these concentrations were 27 μM for *S. vulgaris* and *T. arvense*, whereas in *A. wilhelmsii* (both M and NM), even the highest exposure level (243 μM) did not significantly decrease shoot DW (Fig. 3, Table 2).

Based on the effects of the 243-μM treatment levels of Sb (III) and Sb(V) on root elongation Sb(III) was significantly more toxic than Sb(V) in all the species ($p < 0.001$). However, there was a significant species x treatment [= Sb(III) *versus* Sb(V)] interaction, due to the fact that M and NM *A. wilhelmsii* performed about 10 times better, *T. arvense* about 5 times, and *S. vulgaris* no more than 2 times better under 243 μM Sb(V), in comparison with Sb(III) (Table 2). Based on root or

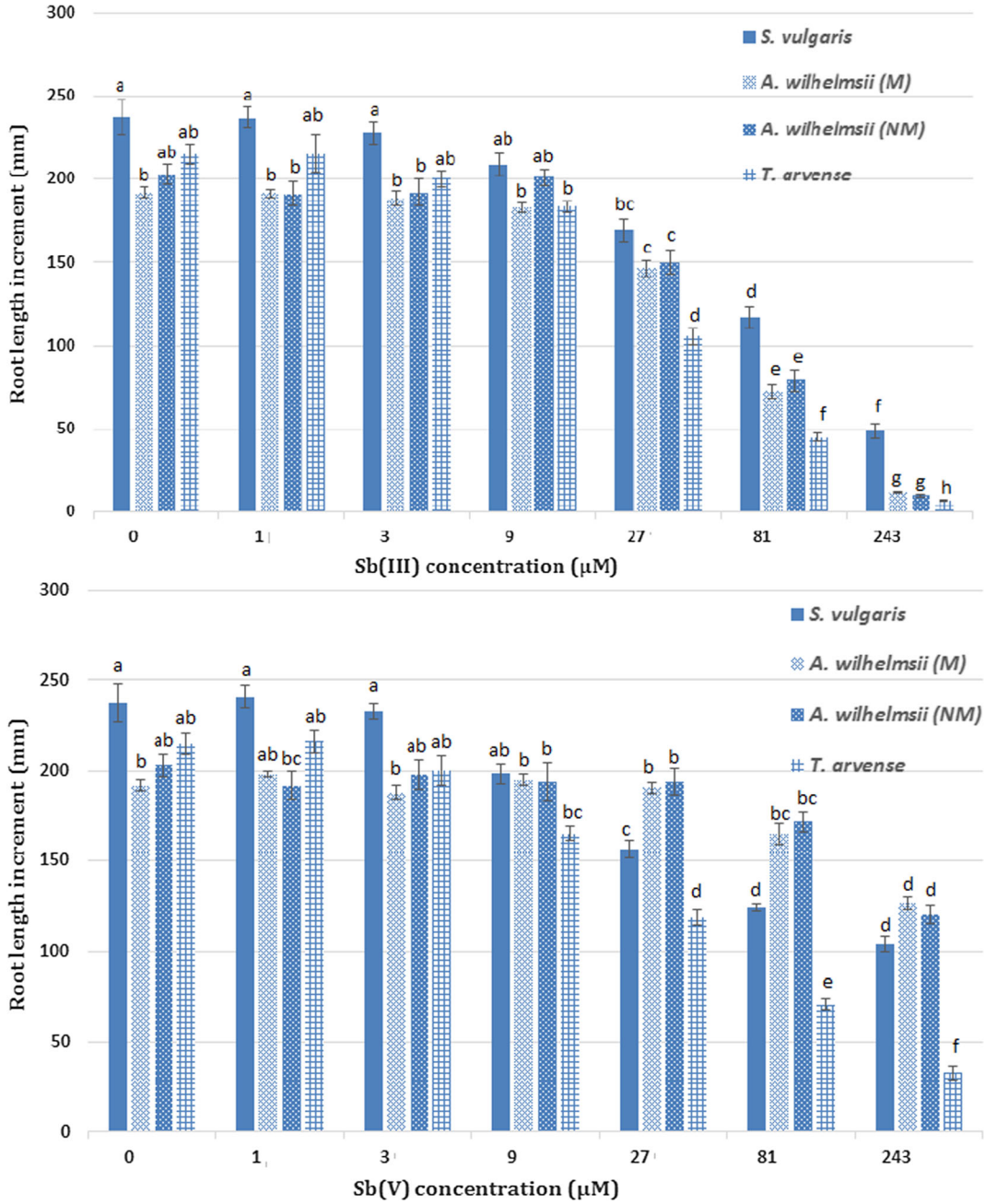

Fig. 1 Effect of different concentrations of Sb(III) and Sb(V) on the root length increment (mean ± SE) in *A. wilhelmsii*, *T. arvense*, and *S. vulgaris*. *Different letters* indicate significant differences ($p < 0.05$) between means

shoot DW, however, both *S. vulgaris* and *T. arvense* did not significantly differently respond to Sb(III), in comparison with Sb(V), whereas M and NM *A. wilhelmsii* showed significantly higher tolerance to Sb(V), with about 2-fold higher root and shoot dry weights, in comparison with the 243-μM Sb(III) treatment (Table 2).

Antimony uptake by the three plant species increased with the Sb concentration in the nutrient solution (Figs. 4 and 5). Both in the Sb(III) and the Sb(V) treatment, there were no considerable differences in Sb root or shoot concentrations between species or *A. wilhelmsii* populations. In all the species Sb uptake was much higher when Sb was supplied as Sb(III), in

Table 1 Lowest Sb(III) and Sb(V) concentrations (μM) in the nutrient solution, at which root elongation, or root or shoot dry weight accumulation were significantly ($p < 0.05$) inhibited, in comparison with the control (lowest EC), in *S. vulgaris*, M and NM *A. wilhelmsii*, and *T. arvense*. Plants were exposed to 0, 1, 3, 9, 27, 81, or 243 μM Sb(III) or Sb (V)

	Root elongation		Root dry weight		Shoot dry weight	
	Sb(III)	Sb(V)	Sb(III)	Sb(V)	Sb(III)	Sb(V)
S. vulgaris	27	27	27	27	81	27
A. wilhelmsii M	27	243	27	243	81	>243
A. wilhelmsii NM	27	243	27	243	81	>243
T. arvense	27	27	3	3	9	27

comparison with Sb(V). In all the species and at all the exposure levels, the root and shoot Sb concentrations were considerably higher in the Sb(III) treatment than they were in the Sb(V) treatment (Fig. 4).

In all of the plant species, both in the Sb(III) and Sb(V) treatments, the shoot Sb concentrations were much lower than the root Sb concentrations, irrespective of the Sb concentration in the nutrient solution.

Since Sb(III) is expected to be spontaneously oxidized to Sb(V) in the presence of molecular oxygen, and since plant roots might be able to reduce Sb(V) to Sb(III), either before or after its uptake into root cells, the time course of Sb(III) oxidation was recorded in pots without and with plants (*A. wilhelmsii*, metallicolous ecotype, three per pot) (Fig. 6). As expected, in pots without plants, the Sb(III) concentration continuously decreased more or less exponentially with time. In pots with plants, however, the Sb(III) concentrations decreased more slowly, tending to stabilize after 4 days, showing that plant roots do reduce Sb(V). In all cases the total Sb concentrations (Sb[III] + Sb[V]) did not significantly change throughout the experiment (data not shown). In pots supplied with 9 or 27 μM Sb(V), there was no detectable Sb(III) accumulation in the solution (data not shown), regardless of the presence of plants. However, when plants grown for three weeks at 243 μM Sb(V) were transferred to 50 mL of Sb-free nutrient solution, after desorbing their roots with 10 mM $CaCl_2$ (10 min), the Sb concentration in the nutrient solution rose to 2.4 (±0.6) μM total Sb at day 2, after which there was no further increase.

To get information regarding the type of transporters involved in Sb(III) and Sb(V) uptake, Sb uptake after 5 days was measured in M *A. wilhelmsii* (metallicolous ecotype), in the presence of various concentrations of nitrate, chloride, and silicon, with a starting concentration of 81 μM Sb(III) or Sb(V) in the nutrient solution. While nitrate was without any effect on either Sb(III) or Sb(V) uptake, silicon drastically inhibited Sb uptake in the Sb(III) treatment, but only slightly in the Sb(V) treatment. In contrast, chloride strongly inhibited Sb uptake in the Sb(V) treatment. In the Sb(III) treatment chloride also decreased Sb uptake, though only slightly, to a barely significant degree ($p = 0.050$) (Fig. 7).

Discussion

Estimated from the effects on root elongation, the high-concentration Sb(III) treatments were clearly more toxic than the corresponding (Sb)V treatments. The lowest concentrations for growth inhibition in our experiment were not different, i.e., 27 μM, except for Sb(V) in M and NM *A. wilhelmsii*, in which root elongation was significantly inhibited not until 243 μM. However, lowest-observed-effect concentrations (LOEC) may

Table 2 Tolerance index (TI) for root elongation and root and shoot dry weights. TI values have been calculated as the means in the 243-μM treatments, divided by the means in the controls, and expressed as % control. Different letters indicate significant differences ($p < 0.05$) between means

	Root elongation		Root dry weight		Shoot dry weight	
	Sb(III)	Sb(V)	Sb(III)	Sb(V)	Sb(III)	Sb(V)
S. vulgaris	20.5 a	43.9 b	40.5 a	41.1 b	29.0 a	35.6 b
A. wilhelmsii M	5.9 b	65.8 a	30.0 ab	63.6 a	26.3 ab	75.6 a
A. wilhelmsii NM	4.9 b	59.1 a	35.1 ab	59.3 a	18.2 ab	72.0 a
T. arvense	3.2 c	15.3 c	19.5 b	19.7 c	11.0 b	10.5 c

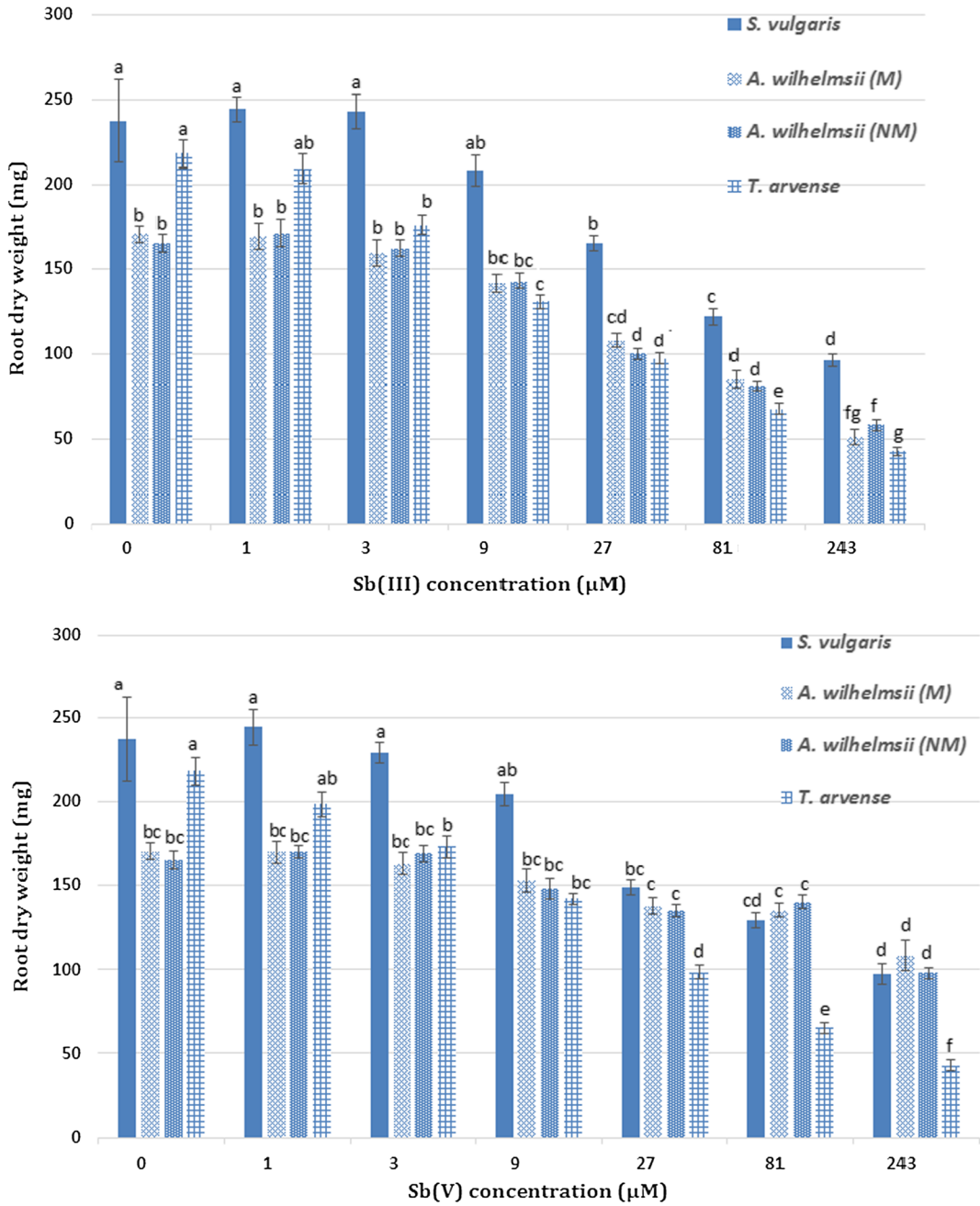

Fig. 2 Root dry weight (mean ± SE) in *A. wilhelmsii*, *T. arvense*, and *S. vulgaris* after 3 weeks of exposure to different concentrations of Sb(III) or Sb(V). *Different letters* indicate significant differences ($p < 0.05$) between means

not sensitively reflect interspecific differences in tolerance. When estimated from root and shoot DW, Sb(III) was only slightly more toxic than Sb(V) in M and NM *A. wilhelmsii*, but not at all in *S. vulgaris* and *T. arvense,* which did not significantly differently respond to Sb(III), in comparison with Sb(V). To distinguish Sb(III) tolerance levels between species, root elongation was clearly the most sensitive end point, yielding significant differences between all the species under study, whereas root or shoot DW identified *T. arvense* as significantly less Sb(III)-tolerant than the others, but did not yield a significant difference between *S. vulgaris* and *A. wilhelmsii*. Regarding Sb(V) tolerance, all the test end points yielded significant

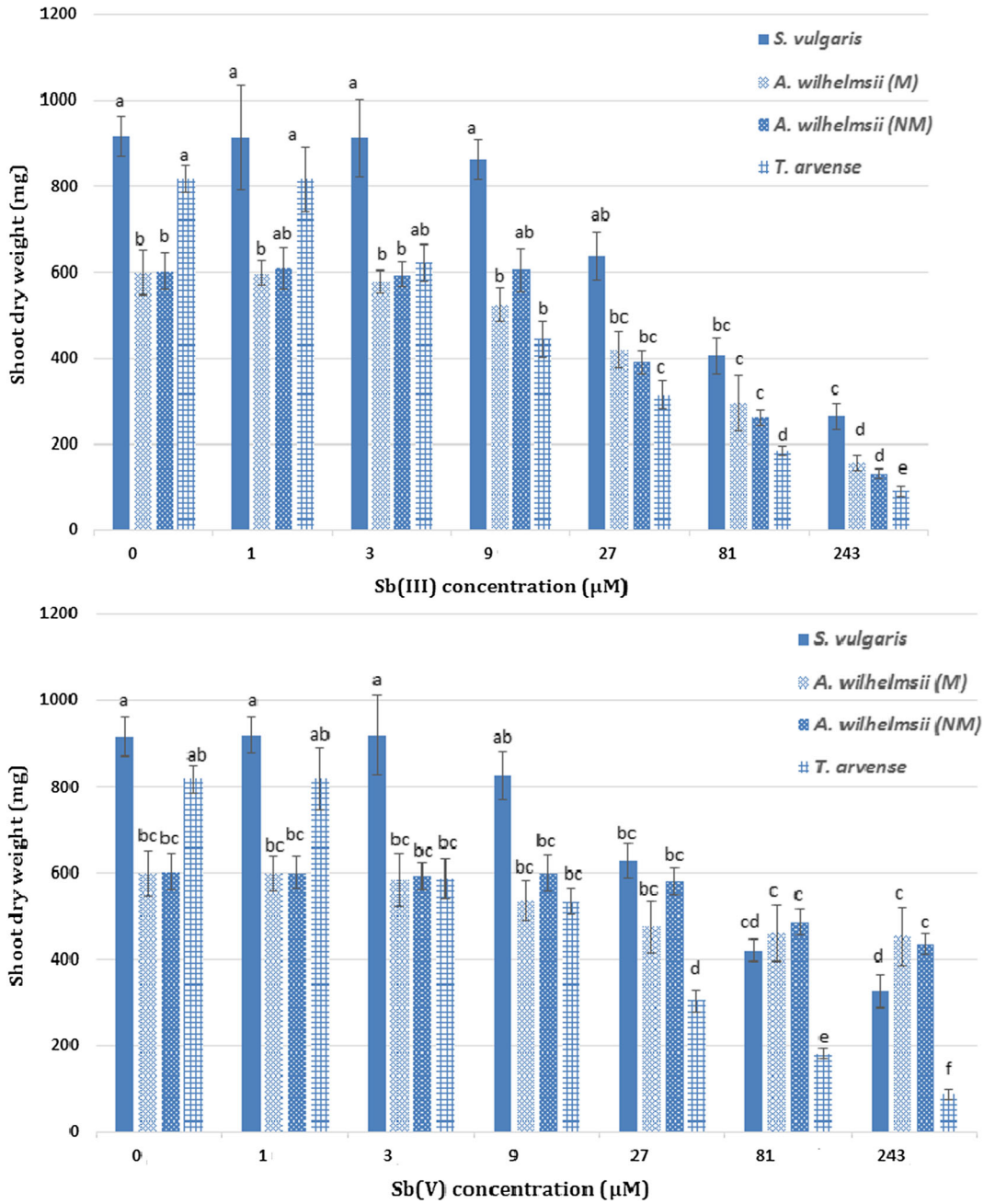

Fig. 3 Shoot dry weight (mean ± SE) in *A. wilhelmsii*, *T. arvense*, and *S. vulgaris* after 3 weeks of exposure to different concentrations of Sb(III) or Sb(V). *Different letters* indicate significant differences ($p < 0.05$) between means

differences between all the species under study. However, it seems that there were differences between species regarding the sensitivities of the different test end points. For example, in *T. arvense* the LOEC for root DW was, both for Sb(III) and Sb(V), 3 μM, which is much lower than that for inhibition of root elongation, i.e., both for Sb(III) and Sb(V), 27 μM (Table 1). In M and NM *A. wilhelmsii*, on the other hand, the LOEC for root DW was much higher than that for root elongation, albeit exclusively for Sb(III) (Figs. 1 and 2). The LOEC values for shoot DW were higher than those for root DW, except for Sb(V) in *S. vulgaris* (Table 1). The relatively low responsiveness of shoot DW, in comparison with that of root DW, might be due to the low Sb

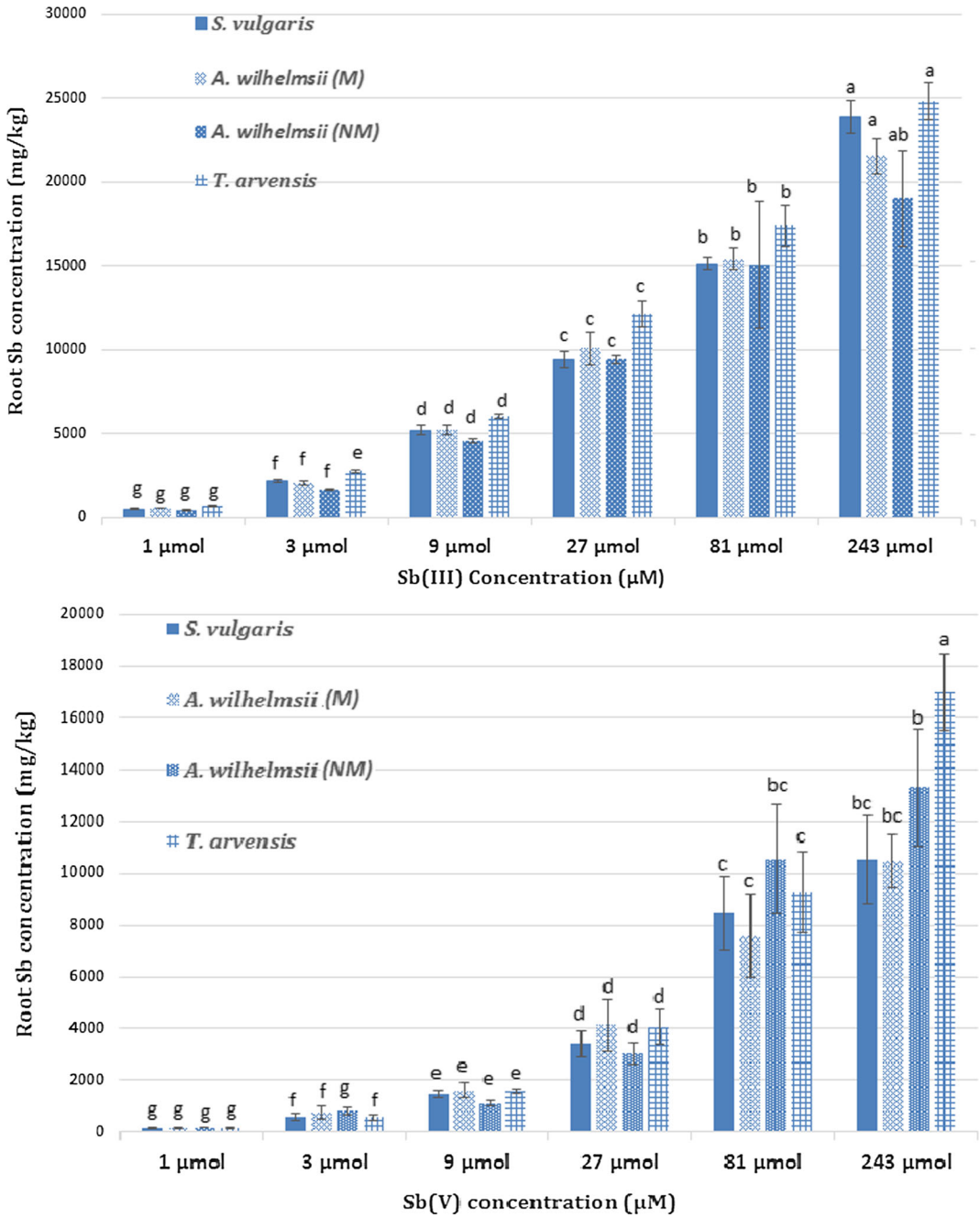

Fig. 4 Root Sb concentrations (mean ± SE) in *A. wilhelmsii*, *T. arvense*, and *S. vulgaris* after 3 weeks at different concentrations of Sb(III) or Sb(V) in the nutrient solution. *Different letters* indicate significant differences ($p < 0.05$) between means

root-to-shoot translocation rates, both in Sb(III) and Sb(V)-treated plants (Figs. 4 and 5).

As argued above, we conclude that Sb(V) tolerance decreases in the order of *A. wilhelmsii* (M/NM) > *S. vulgaris* > *T. arvense*, whereas Sb(III) tolerance decreases in the order of *S. vulgaris* > *A. wilhelmsii* (M/NM) > *T. arvense*, albeit that the difference in the Sb(III) tolerance index between *S. vulgaris* and *A. wilhelmsii* is exclusively significant when root elongation is used as a test end point. Thus, of all the species, *T. arvense* is clearly the most sensitive one, both to Sb(III) and Sb(V), whereas *S. vulgaris* is more Sb(III)-

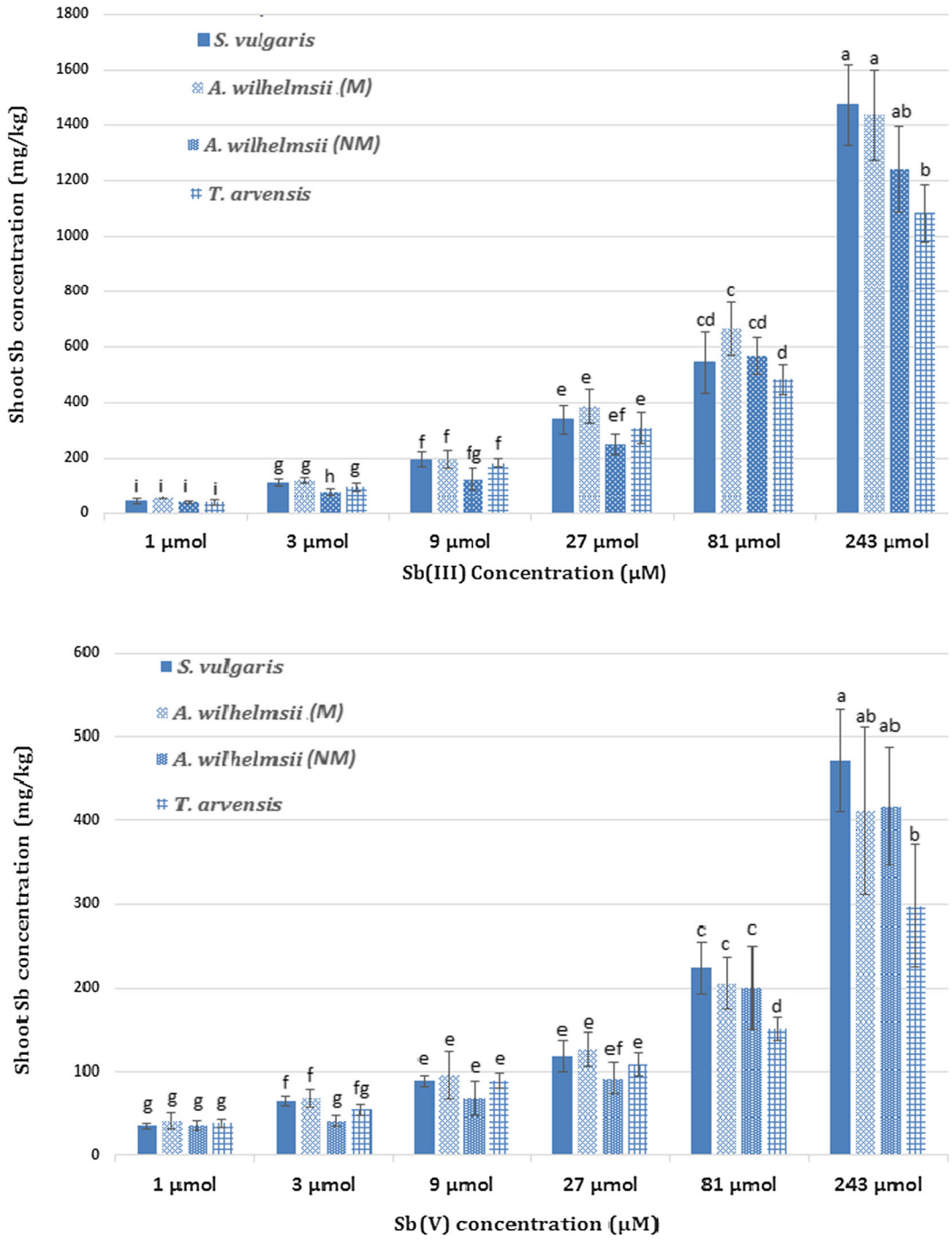

Fig. 5 Shoot Sb concentrations (mean ± SE) in *A. wilhelmsii*, *T. arvense*, and *S. vulgaris* after 3 weeks at different concentrations of Sb(III) or Sb(V) in the nutrient solution. *Different letters* indicate significant differences ($p < 0.05$) between means

tolerant than *A. wilhelmsii*, and *A. wilhelmsii* is much more Sb(V)-tolerant than *S. vulgaris*. In other words, the interspecific variation in Sb(III) tolerance appears to be uncorrelated with that in Sb(V) tolerance. In view of fact that there was no considerable interspecific variation in Sb accumulation in roots and shoots, regardless of whether Sb was supplied as Sb(III) or Sb(V) (Figs. 4 and 5), it is plausible to assume that the interspecific

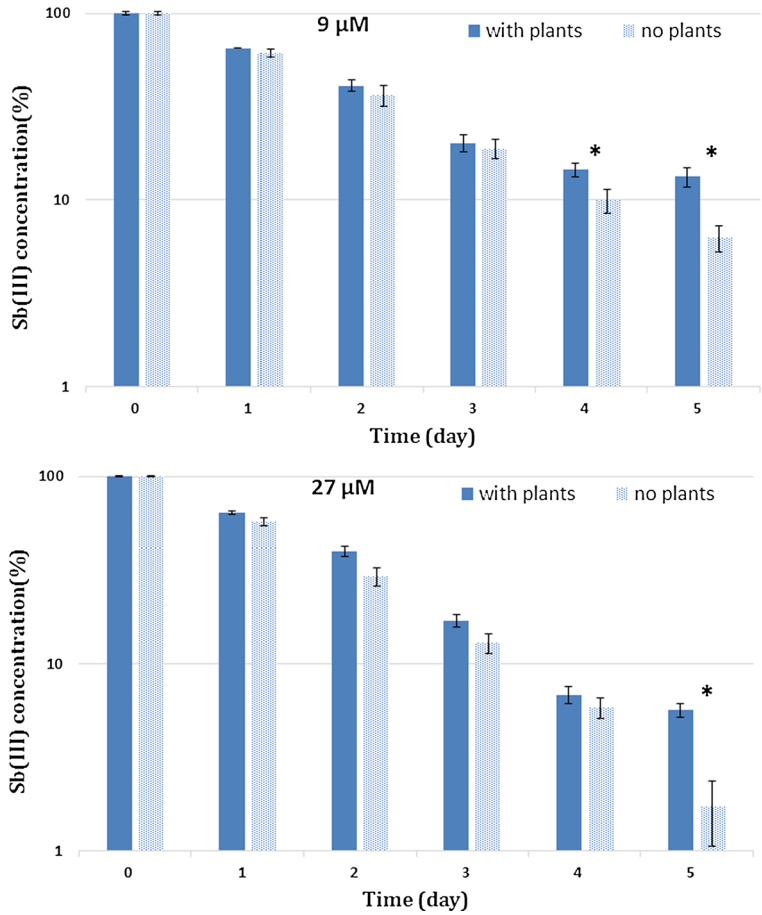

Fig. 6 Change of Sb(III) concentration (mean ± SE) in the nutrient solution with and without metallicolous *A. wilhelmsii*, in the 9-μM and 27-μM treatments. *Significant difference ($p < 0.05$) between pots with and pots without plants

variation in both Sb(III) tolerance and Sb(V) tolerance relies on differential capacities to sequester Sb inside the plant body, particularly the root. Moreover, since Sb(III) tolerance and Sb(V) tolerance vary independently, it seems that plants use different mechanisms for Sb(III) and Sb(V) sequestration, with independently varying capacity limits.

The Sb accumulation rates in roots and shoots were about 3-fold higher in the Sb(III) treatment, in comparison with the Sb(V) treatment. This implies that the rate of Sb(III) uptake must in fact have been more than threefold higher than that of Sb(V), because Sb(III) is rapidly oxidized in the nutrient solution, until it reaches a concentration in the low micro-molar range, far below the nominal concentrations supplied (Fig. 6). Moreover, since plant roots are apparently able to reduce root-external Sb(V) to Sb(III), it is conceivable that part of the Sb taken up in the Sb(V) treatment can have been taken up as Sb(III). However, since Sb(III) did not accumulate at detectable concentrations in Sb(V)-amended nutrient solutions, and given the relatively short half-life of Sb(III) in the nutrient solution (±1.25 d), it can be safely assumed that the bigger part of the Sb taken up in the Sb(V) treatment must have been taken up as Sb(V). Conversely, since Sb accumulation is about 3-fold higher in the Sb(III) treatment than in the Sb(V) treatment, at least two-third of the Sb burden of the Sb(III)-treated plants must have been taken up as Sb(III). This means that, on a plant-internal basis, Sb(V) is probably more toxic than Sb(III), possibly except for *A. wilhelmsii*. For example, when present at 27 μM in the nutrient solution, Sb(III) and Sb(V) are about equally toxic in *S. vulgaris* and *T. arvense*, both in terms of root elongation inhibition and root DW reduction (Figs. 1 and 2). However, in both species the root-internal Sb concentrations are about 3-fold higher in the Sb(III) treatment than they are in the Sb(V) treatment. Thus, although plant-external Sb(III) is taken up at a much higher rate and, therefore, usually more toxic than plant-external Sb(V), plant-internal Sb may in fact be

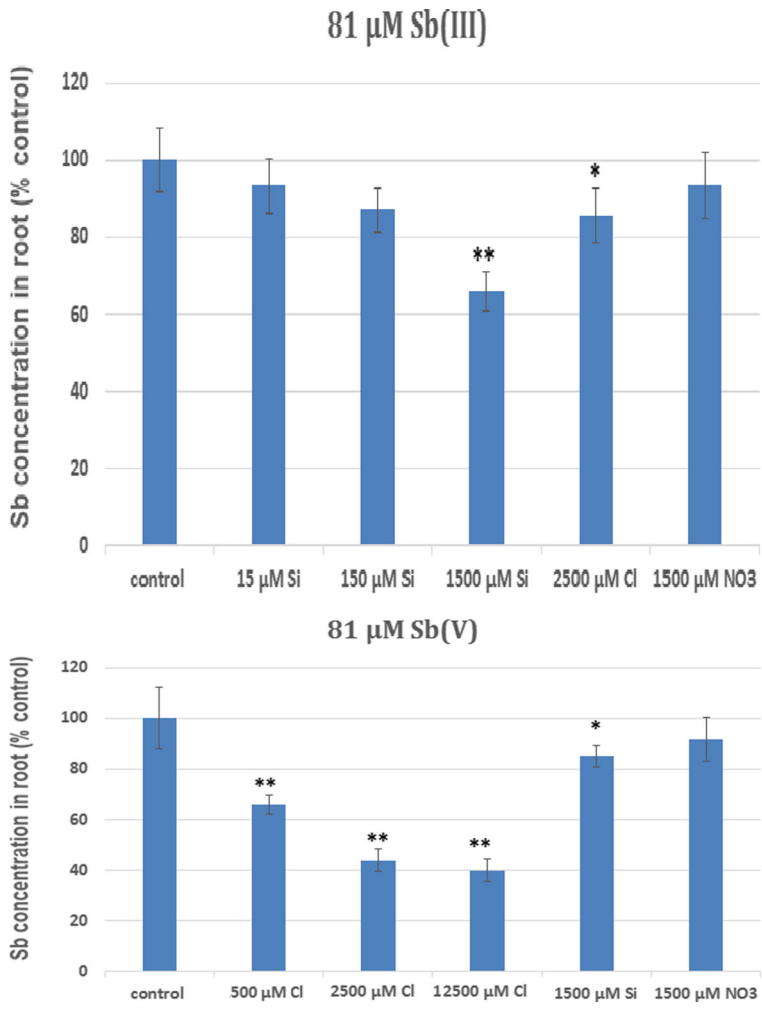

Fig. 7 Effect of different concentrations of silicon, chloride and nitrate on root Sb concentrations (mean ± SE) after 5 d exposure to 81 µM Sb(III) or Sb(V). *significantly different from the control ($p < 0.05$); **significantly different from the control ($p < 0.01$)

more toxic when it is taken up as Sb(V). For example, when compared at approximately equal root-internal Sb concentrations, *S. vulgaris* [e.g., at 243 µM Sb(V) and 27 µM Sb(III)] and *T. arvense* [e.g., at 243 µM Sb(V) and 81 µM Sb(III)] suffer more from Sb(V)-imposed toxicity than from Sb(III)-imposed toxicity, both when estimated from root elongation and root DW (Figs. 1 and 2). In *A. wilhelmsii*, on the other hand, when compared at equal root-internal Sb concentrations [e.g., 243 µM Sb(V) and 27 µM Sb(III)], there is no significant difference between the effects of Sb(V) and Sb(III) exposure (Figs. 1 and 2), suggesting once more that the higher Sb(V) tolerance in this species, in comparison with *S. vulgaris* and *T. arvense*, must rely on an enhanced capacity to sequester, specifically, Sb(V).

The detoxification in plant cells of Sb(III), which is a strong inducer of phytochelatins, is most probably based on chelation by phytochelatins (Wysocki et al. 2003; Le Faucheur et al. 2006), presumably followed by transport of the Sb(III)-phytochelatin complex into the vacuole, mediated by ABCC-type transporters, exactly as its chemical analogue, arsenite (Song et al. 2010). The mechanism of Sb(V) detoxification in plants has not been explored yet. Based on the observation that plant roots are apparently able to reduce Sb(V) to Sb(III) (this study), one might expect that Sb(V) would follow the detoxification pathway of As(V), which consists of reduction to As(III) by arsenate reductases (Bleeker et al. 2006; Chao et al. 2014), followed by either storage as As(III)-phytochelatin complexes in the vacuole (Bleeker et al. 2006; Song et al. 2010), or efflux from the roots, as H_3AsO_3 (Xu et al. 2007; Zhao et al. 2010; Chao et al. 2014). However, although we found evidence of Sb efflux from roots, presumably as antimonite, the exudation rate was inconsiderable, in comparison with that of arsenite (Xu et al. 2007; Zhao

et al. 2010; Chao et al. 2014). Our results also showed that Sb(III) does not accumulate at detectable concentrations in nutrient solutions with lower Sb(V) concentrations, suggesting that plant roots can only efflux considerable parts of their Sb burden when Sb is supplied as Sb(III), such as demonstrated in *Pteris vittata* (Tisarum et al. 2014). Moreover, although only two reports on Sb(V)-induced phytochelatin accumulation or phytochelatin-dependent Sb(V) tolerance are available thus far, results were negative in both case studies, even though there was considerable Sb accumulation (Le Faucheur et al. 2006; Corrales et al. 2014). Moreover, Sb(V)-exposed plants store Sb largely as Sb(V), rather than Sb(III) (Tisarum et al. 2014; Ren et al. 2014). This suggests that the reductive detoxification pathway may not be very important for Sb(V). In contrast, the detoxification of As(V) relies largely on reduction, followed by efflux, or PC-dependent sequestration, as shown by the enhanced As accumulation and As(V) sensitivity of As(V) reductase- or phytochelatin-deficient mutants (Bleeker et al. 2006; Zhao et al. 2010; Chao et al. 2014).

Our results confirm that Sb(III), like As(III), can be taken up via silicon-permeable aquaporins, such as Lsi1 (Ma et al. 2008). Sb(V) uptake, on the other hand, is effectively suppressed by chloride, but not by nitrate. Meanwhile, it has been shown that Sb(V) uptake is also unaffected by phosphate (N Jamali Hajiani and SM Ghaderian, unpublished), in contrast to that of As(V) (Meharg and Macnair 1994). The latter is not surprising, in view of the completely different speciation of Sb(V) and As(V), viz. $SbO_6H_6^-$, and $AsH_2O_4^-$ or $AsHO_4^{2-}$, respectively. It is not clear in which form Sb is translocated to the shoot. In any case, in our study the Sb concentrations in the shoot are more than one order of magnitude lower than those in the roots in all of the species, regardless of whether Sb was supplied as Sb(III) or Sb(V).

If Sb(III) sequestration would depend on phytochelatin synthesis indeed (see above), then it is not surprising that *S. vulgaris* is the most Sb(III)-tolerant one of all the species. *S. vulgaris* seems to have an exceptionally high capacity for phytochelatin synthesis, particularly when under Cd or As exposure (De Knecht et al. 1994; Sneller et al. 1999; Schat et al. 2002).

A. wilhelmsii is by far the most Sb(V)-tolerant species. Remarkably, there is no difference in tolerance to both Sb(V) and Sb(III) between the metallicolous and the non-metallicolous population of this species, suggesting that high-level Sb(V) tolerance is a species-wide property in *A. wilhelmsii*. Given that both populations originate from dry, oxidized soils, it is not surprising that there is no difference in their tolerance to Sb(III), to which they are normally not exposed in nature. However, it is reasonable to expect a higher Sb(V) tolerance in the mine population, in comparison with the non-metallicolous reference population, such as generally found for non-ferrous metal mine populations of the majority of facultative metallophytes, (Antonovics et al. 1971; Schat et al. 2002), due to repeated independent micro-evolutionary adaptation at a local scale (Schat et al. 1996). The reason for the apparent absence of local evolutionary adaptation in the metallicolous *A. wilhelmsii* population might simply be that it does not suffer from Sb toxicity. This is also indicated by the fact that this population accumulates Sb to, on average, ± 150 mg/kg DW Sb in its leaves in nature (Jamali Hajiani et al. 2015), which is only marginally higher than it did in this study at the highest non-toxic Sb(V) exposure level (81 μM). In addition, cases of Sb hypertolerance in populations from Sb-enriched soil, in comparison with conspecific non-metallicolous populations, have never been reported thus far. This could mean that the phenomenon has been insufficiently explored yet. Alternatively, it could also mean that, even in strongly Sb-enriched soils, Sb, or at least Sb(V), may in fact not be phytotoxic for many species, regardless of whether they are Sb-adapted or not. In line with this, Corrales et al. (2014), studying two non-metallophyte clover species, did not find any toxicity symptoms even at 200 μM Sb(V) in the nutrient solution, while the foliar Sb concentrations were about 400 and 800 mg/kg DW, respectively. However, further research is needed to get a better picture of the variation in Sb susceptibility among and within plant species, and the frequency of, whenever it occurs at all, micro-evolutionary adaptation to Sb-enriched soil.

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