



## Research article

Effects of antimony on enzymatic and non-enzymatic antioxidants in a metalicolous and a non-metallicolous population of *Salvia spinosa* LShakiba Rajabpoor<sup>a</sup>, Seyed Majid Ghaderian<sup>a,\*</sup>, Henk Schat<sup>b</sup><sup>a</sup> Department of Plant and Animal Biology, Faculty of Biological Science and Technology, University of Isfahan, Isfahan, 81746-73441, Iran<sup>b</sup> Department of Ecological Science, Faculty of Sciences, Vrije Universiteit, De Boelelaan 1085, 1081 HV, Amsterdam, the Netherlands

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## ABSTRACT

The present study aimed at elucidating the role of antioxidants and stress metabolites in antimony (Sb) tolerance in a metalicolous (M), Sb[V]-hypertolerant population, and a non-metallicolous (NM) population of *Salvia spinosa*, particularly with regard to the question of whether they could be involved in constitutive Sb tolerance or, specifically, in Sb[V] hypertolerance in the M population. Plants were exposed in hydroponics to 0, 8, 24, 74, 221  $\mu\text{M}$  Sb (III or V). Superoxide dismutase (SOD), ascorbate peroxidase (APX) and catalase (CAT) activities, and the concentrations of phenolics, flavonoids, and proline in leaves were measured after 20 d. As potential stress/tolerance markers, the concentrations of chlorophyll *a* and *b*, anthocyanins, and those of total soluble and reducing sugars were also measured. Chlorophyll *a* concentration reflected the difference, both in Sb[III] and Sb[V] tolerance, between N and NM, and the higher toxicity of Sb[III], compared to Sb[V]. APX and proline accumulation were more induced in M than in NM, and more by Sb[V] than by Sb[III], which is theoretically compatible with a role in Sb[V] hypertolerance. CAT was more induced in M than in NM, but more by Sb[III] than Sb[V], suggesting that is not functional in Sb[V] hypertolerance. The other enzymes and compounds did not exhibit significant Sb redox status\*population interactions, suggesting that they don't play a role in, specifically, Sb[V] hypertolerance in M, but at most in the constitutive Sb[III] or Sb[V] tolerance of the species.

## 1. Introduction

Environmental contamination by heavy metals and metalloids is a major problem. One of the most toxic metalloids is antimony (Sb). Antimony (atomic number 51; atomic weight 121.76) is the ninth most-mined metalloid, and causes increasing environmental contamination (Wang et al., 2015). In the environment Sb is present as either antimonite (Sb[III]), or antimonate (Sb[V]), dependent on the soil redox status. Inorganic Sb species have been found to be more toxic than organic ones (Filella et al., 2002), and Sb[III] is more toxic than Sb[V] (Jamali Hajiani et al., 2017; Rajabpoor et al., 2019). The Sb concentration in normal soil is about 0.3–8.4 mg/kg (Tschan et al., 2009).

Plants can absorb different Sb forms, and for each form, the rates of absorption and root-to-shoot transfer and tolerance vary considerably among plant species (Jamali Hajiani et al., 2017), or even among conspecific populations (Rajabpoor et al., 2019). Both inorganic Sb forms, antimonite and antimonate, can be taken up by plants, most probably by some phosphate transporter(s) (Feng et al., 2013), and silicon transporter(s) (Jamali Hajiani et al., 2017), or at least the nodulin 26-like intrinsic protein NIP1; 1 (Kamiya and Fujiwara, 2009),

respectively. Exposure to Sb causes physiological changes, such as inhibition of growth and development, suppression of photosynthesis, decreased synthesis of several metabolites, enhanced activities of reactive oxygen species, and disturbance of nutrient absorption (Tschan et al., 2009; Karacan et al., 2016).

Like several other heavy metals and metalloids, Sb is a potent inducer of ROS accumulation (Feng et al., 2009). Although several ROS species can induce protective responses to a broad array of stressors, including heavy metals and metalloids, excessive ROS accumulation eventually results in 'oxidative stress' (Molassiotis et al., 2006). To protect themselves against oxidative stress, plants produce enzymatic, as well as non-enzymatic antioxidants (Ahsan et al., 2009). It has been suggested that enhanced activities of peroxidase (POD), catalase (CAT) and ascorbate peroxidase (APX) are component parts of the Sb[III] tolerance mechanism in Sb[III]-tolerant plants (Pan et al., 2011). Ascorbate and glutathione, as well as phenolics and related compounds, such as flavonoids, phenylpropanoids, glycosides and carotenoids are examples of non-enzymatic antioxidants (Sharma et al., 2012). Polyphenols can directly eliminate ROS, and inhibit lipid peroxidation of membranes by scavenging lipid alkoxyl radicals (Sharma et al., 2012).

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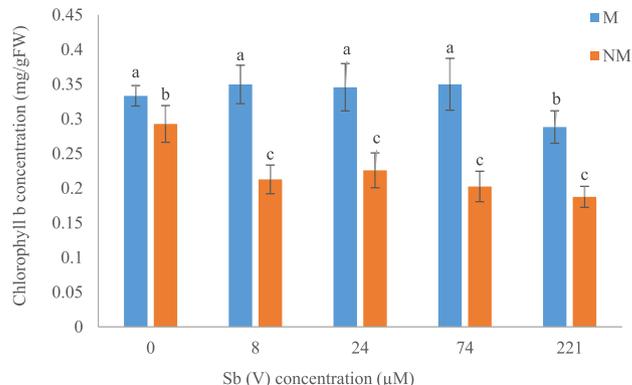
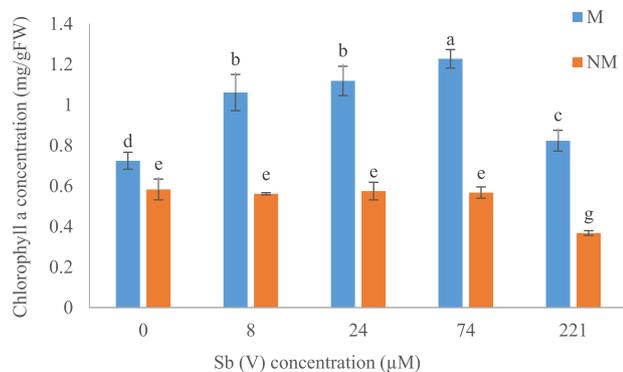
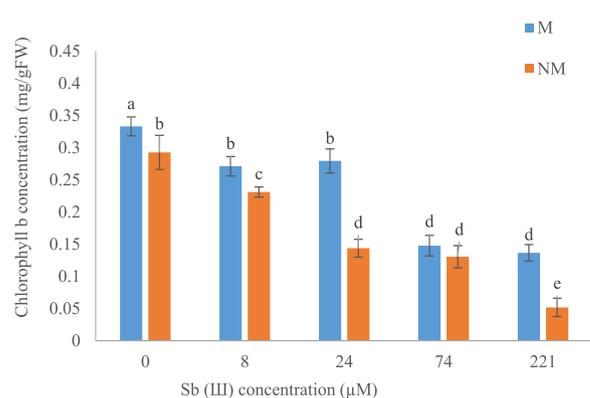
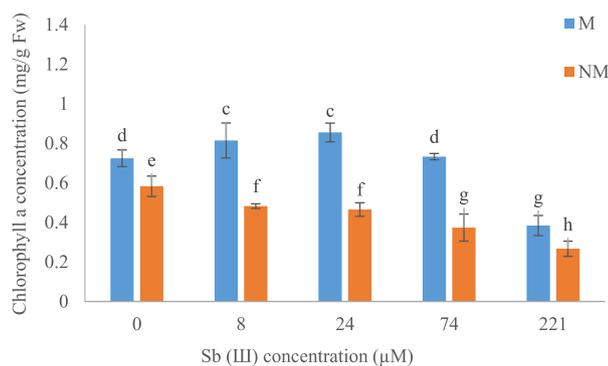
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**Table 1**

Probabilities (p) of the main effects (Sb[III] or Sb[V] and population [NM versus M]) and their interactions in the Sb[III] treatment (columns 1–3), the Sb[V] treatment (columns 4–6), and the effects of the Sb redox status (Sb[III] versus Sb[V]) and the Sb redox status\*population interaction (columns 7, 8). \*\*\* =  $p < 0.001$ ; \*\* =  $p < 0.01$ ; \* =  $p < 0.05$

	Sb[III]	pop.	Sb[III]*pop.	Sb[V]	pop.	Sb[V]*pop.	Sb red.	Sb red*pop.
Chlorophyll a	***	***	***	***	***	***	***	*
Chlorophyll b	***	***	***	***	***	***	***	ns
Chlorophyll a + b	***	***	***	***	***	***	***	< 0.1
Anthocyanins	***	**	***	***	***	***	***	ns
Proline	***	***	***	***	***	***	***	< 0.1
Phenolics	***	***	***	***	***	***	***	ns
Flavonoids	***	*	***	***	***	***	***	ns
Catalase	***	**	***	***	ns	***	***	***
Ascorbate peroxidase	***	*	*	***	***	***	***	***
Superoxide dismutase	***	***	***	***	***	***	***	ns
Water-soluble sugars	***	***	**	***	***	ns	*	ns
Reducing sugars	***	***	ns	***	***	ns	**	ns



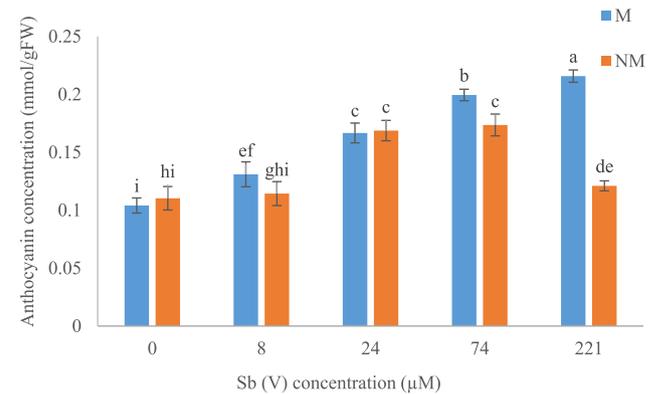
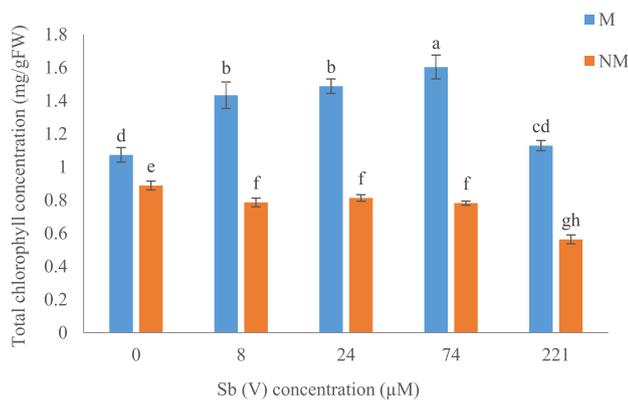
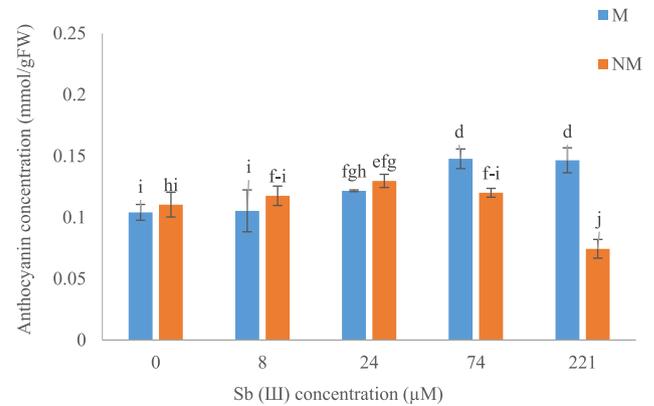
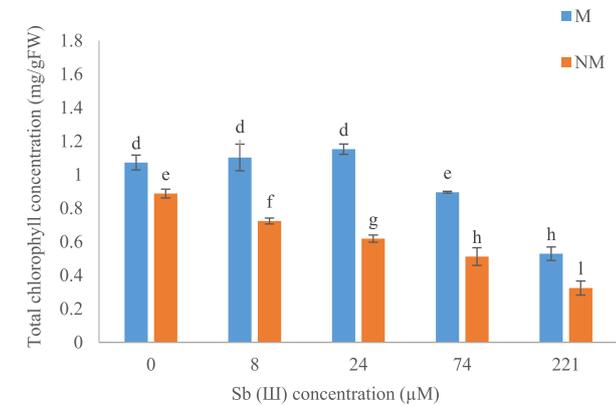
**Fig. 1.** Chlorophyll a content (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb[III] or Sb[V] in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

**Fig. 2.** Chlorophyll b content (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb[III] or Sb[V] in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

The observed increase of phenolic compounds, especially flavonoids, in response to Sb exposure suggests that these compounds might protect the plant against Sb toxicity, or Sb-induced oxidative stress at least (Ortega et al., 2017).

Metal-imposed induction of enzymatic and non-enzymatic antioxidant capacities are considered to contribute to metal tolerance (Gill and Tuteja, 2010). However, in many case studies it is not clear whether they contribute to constitutive tolerance or hypertolerance. In case of ‘facultative metallophytes’, with metalicolous and non-metalicolous populations, it has often been observed that the threshold exposure levels for induction of enhanced antioxidant capacities or accumulation

of ‘stress metabolites’ were much higher in hypertolerant plants than they were in non-metalicolous plants, such as demonstrated for proline accumulation in *Silene vulgaris* (Schat et al., 1997). Such results may be taken to suggest that antioxidant or stress metabolite induction is primarily a symptom of stress, or a constitutive tolerance mechanism, rather than a mechanism of hypertolerance, and that the slower induction in hypertolerant plants is a mere reflection of their superior tolerance, which shifts the threshold exposure for stress responses to a higher level (Schat and Kalf, 1992; Schat et al., 1997). Of course, this does not mean that induced antioxidant or stress metabolite induction would be non-functional in hypertolerant plants. It might well be



**Fig. 3.** Total chlorophyll content (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb(III) or Sb(V) in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

functional in both non-metallicolous and hypertolerant plants, although hypertolerant plants may be less dependent on these mechanisms, because they have other, more efficient, e.g. transporter-based, mechanisms in addition. Moreover, in some cases, hypertolerant plants have been reported to constitutively express one or more normally stress-responsive genes at high levels (Van de Mortel et al., 2006), suggesting that constitutive expression of particular, normally stress-responsive genes may in fact represent a component part of the hypertolerance mechanism (Ciarmiello et al., 2011).

As recently demonstrated in *Salvia spinosa*, strongly Sb-enriched soils can provoke genetic, 'micro-evolutionary' adaptation, including hypertolerance, to Sb, at least Sb[V], in comparison with 'non-metallicolous' con-specific populations or other plants species (Rajabpoor et al., 2019). The mechanism(s) underlying Sb hypertolerance in *S. spinosa* have not been explored thus far. In this study, we compared the effects of Sb(III) and Sb(V) on some (potential) toxicity markers (chlorophylls, sugars, anthocyanins) and (potential) enzymatic (APX, CAT, SOD) and non-enzymatic antioxidants (phenolics, flavonoids) or osmolytes/osmoprotectants (sugars, proline) in a non-metallicolous and a metallicolous, Sb[V]-hypertolerant population of *S. spinosa*. The primary aim was to assess whether any of these antioxidants or osmoprotectants could contribute to Sb[V] hypertolerance in the metallicolous *S. spinosa* population, or alternatively, merely play a role in the 'constitutive' Sb tolerance in both populations.

**Fig. 4.** Anthocyanin content (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb(III) or Sb(V) in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

## 2. Materials and methods

### 2.1. Plant materials and treatment

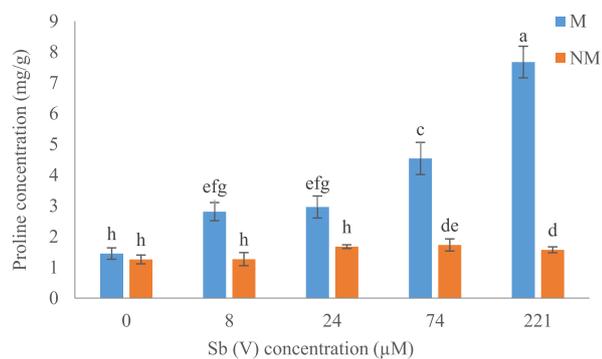
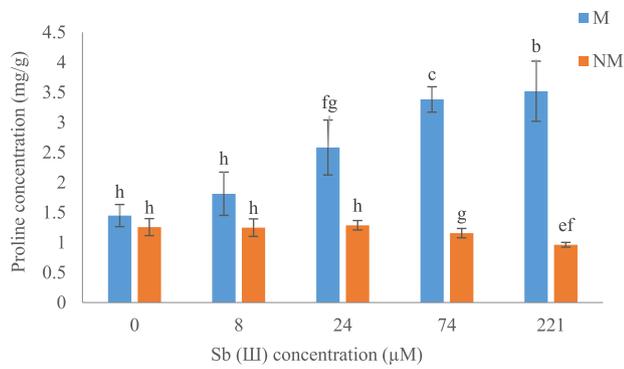
*S. spinosa* seeds were collected from a strongly Sb-enriched site near the Dashkasan mine, and a non-contaminated site in the Chaharmahal area (Rajabpoor et al., 2019, for site descriptions). Seeds were germinated and seedlings were grown in hydroponics in a growth chamber (20/15 °C day/night; light intensity 200  $\mu\text{E m}^{-2} \text{s}^{-1}$ , 14 h day<sup>-1</sup>; relative humidity 75%), exactly as described in Rajabpoor et al. (2019). Plants were exposed for 3 weeks to different concentrations of Sb(III) or Sb(V) (0, 8, 24, 74 and 221  $\mu\text{M}$ , 4 pots per concentration per population). Sb(III) and Sb(V) were supplied as potassium antimonite tartrate tetrahydrate and potassium hexahydroxyantimonate, respectively.

### 2.2. Photosynthetic pigments

Leaves (0.1 g) were homogenized in 80% acetone at 4 °C in the dark. The extract was filtered through Whatman No.1 filter paper. The volume was adjusted with 80% acetone to 10 ml. The absorbance of the extract was read spectrophotometrically at 470, 645 and 663 nm against the solvent blank (80% acetone). The concentrations of chlorophyll *a*, chlorophyll *b* and total chlorophyll concentration were calculated according to Arnon (1949).

### 2.3. Anthocyanin

Anthocyanin was measured following Wagner (1979). In short,



**Fig. 5.** Proline content (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb[III] or Sb[V] in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

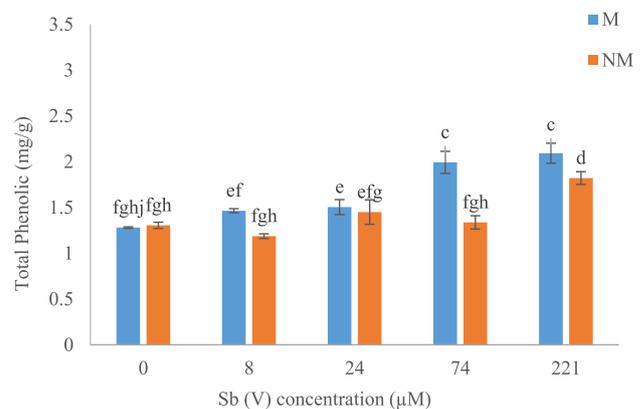
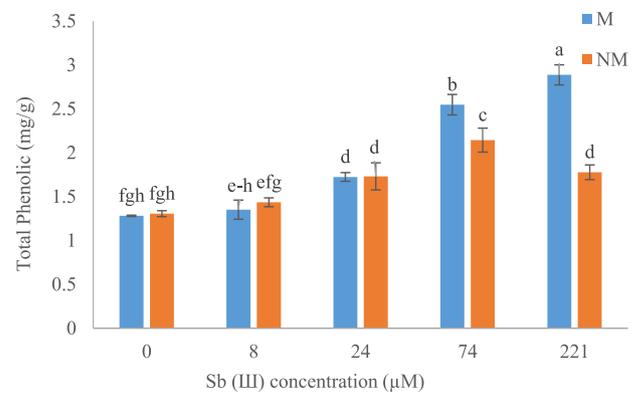
leaves (0.1 g) were homogenized in 10 ml acidified methanol (methanol: HCl, 99:1 [v/v]) and the extract was kept at 25 °C in the dark for 24 h. The homogenate was centrifuged at 4000 rpm for 10 min and the absorbance of the supernatants was determined at 550 nm.

#### 2.4. Proline

Proline was determined according to Bates et al. (1973), based on the reaction of proline with ninhydrin. Fresh leaf material (50 mg) was homogenized with 1.7 ml of 3% sulfosalicylic acid and the homogenate centrifuged at 14,000 rpm for 20 min. The supernatant was mixed with glacial acetic acid and ninhydrin acid in a 1:1:1 ratio. The mixture was placed in a boiling water bath for 1 h. Thereafter, the chromophore was extracted with 2 ml toluene and vortexed for 2 min. Then its absorbance at 490 and 520 nm was determined in a spectrophotometer, using toluene as the blank and L-proline as a standard.

#### 2.5. Phenolics

Fresh leaf material (0.1 g) was homogenized in 80% methanol. The homogenates were adjusted with 80% methanol to a volume of 10 ml and centrifuged for 10 min at 4000 rpm. The Folin-Ciocalteu method was used to measure the total phenol content (Singleton et al., 1999). Briefly, 500  $\mu$ l of crude extract was mixed with 1.5 ml of 10% Folin-Ciocalteu reagent. After 5 min of incubation at room temperature, 1 ml of 7.5% sodium carbonate solution was added. Samples were incubated for 45 min at room temperature. The absorbance at 760 nm was determined. Gallic acid was used as a standard and the results were expressed as milligram of gallic acid equivalents per gram dry weight of



**Fig. 6.** Total phenolics content (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb[III] or Sb[V] in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

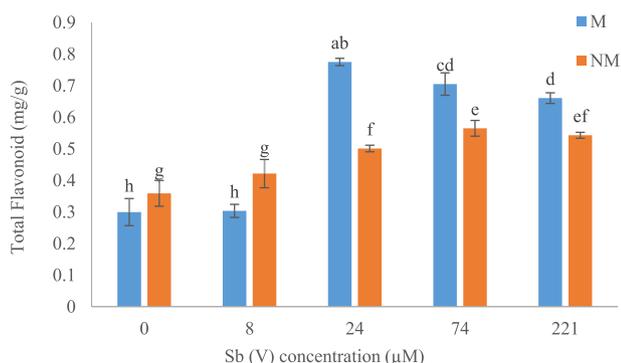
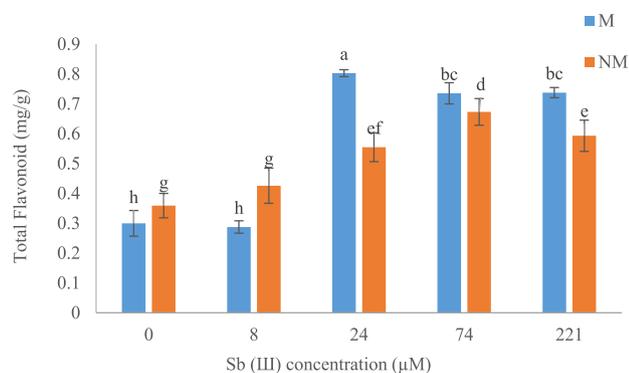
extract (mg GAE/g dw). The concentration of phenolics in various extracts was expressed as GAE/g using standard gallic acid.

#### 2.6. Flavonoids

The total flavonoid content was determined according to the aluminium chloride colorimetric method, using quercetin as standard (Chang et al., 2002). The reaction mixture contained 100  $\mu$ l of crude methanol extract (see above), 200  $\mu$ l 80% ethanol, 200  $\mu$ l 1 M potassium acetate and 200  $\mu$ l 10% aluminium chloride hexahydrate. The mixture was incubated for 30 min at room temperature and the absorbance of the reaction mixture was measured at 415 nm. Then the total flavonoid content was calculated using the standard curve of quercetin.

#### 2.7. Protein extraction and enzyme activity

Fresh leaf samples (100 mg) were homogenized in 3 ml of 50 mM potassium phosphate buffer (pH 7.8), containing 0.2 mM EDTA, 2% (w/v) polyvinylpyrrolidone (PVP), 4 mM dithiothreitol (DTT) and 5 mM magnesium sulphate, 10% glycerol and 2% (w/v) polyvinylpyrrolidone, using mortar and pestle. The homogenate was centrifuged at 12,000 g for 20 min at 4 °C. Protein concentrations were determined according to Bradford (1976). The calibration curve was made with BSA. The activities of catalase (CAT), ascorbate peroxidase (APX) and superoxide dismutase (SOD) were determined in fresh extracts.



**Fig. 7.** Total flavonoids content (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb[III] or Sb[V] in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

### 2.7.1. Catalase (CAT)

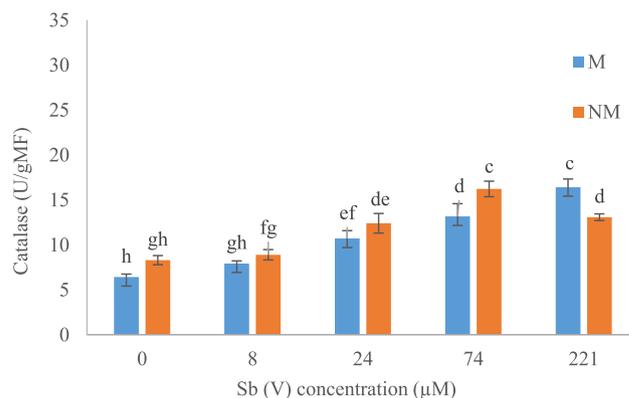
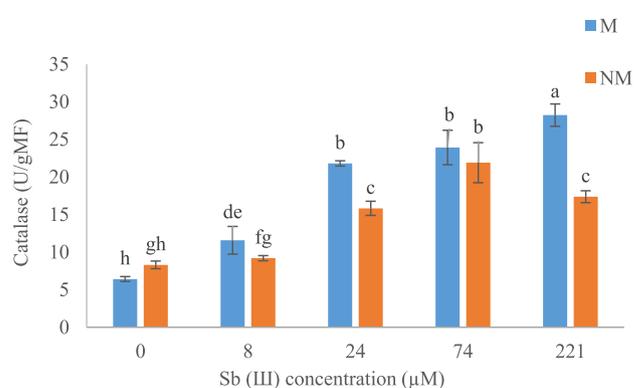
Catalase activity (EC.1.11.1.6) was assayed according to Aebi (1984). Protein extract (100  $\mu$ l) was added to 900  $\mu$ l reaction solution (5 mM  $H_2O_2$  in 50 mM potassium phosphate buffer, pH 7). The absorbance of  $H_2O_2$  was measured at 240 nm, 60 s after starting the reaction. One unit of catalase activity is defined as the amount of enzyme that is needed for the decomposition of 1  $\mu$ mol of  $H_2O_2$  in 60 s at 25  $^{\circ}C$ . Enzyme activity was calculated from the extinction coefficient ( $\epsilon = 0.039 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

### 2.7.2. Ascorbate peroxidase (APX)

APX Activity (EC 1.11.1.11) was determined following Nakano and Asada (1981). The reaction solution (900  $\mu$ l) was a mixture of 725  $\mu$ l of 50 mM potassium phosphate buffer (pH 7) with 0.2 mM EDTA, and 175  $\mu$ l of 0.5 mM ascorbic acid. Supernatant (50  $\mu$ l) was added to 900  $\mu$ l of reaction buffer and then 50  $\mu$ l of 200 mM  $H_2O_2$  was added to start the reaction. The decrease in absorbance at 290 nm was recorded during 60 s upon  $H_2O_2$  addition. One unit of ascorbate peroxidase is defined as the oxidation of 1  $\mu$ mol  $\text{min}^{-1}$  ascorbic acid at 25  $^{\circ}C$ . Enzyme activity was calculated from the extinction coefficient ( $\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$ ).

### 2.7.3. Superoxide dismutase (SOD)

SOD activity (EC 1.15.1.1) was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium (NBT), as described by Giannopolitis and Ries (1977). The reaction mixture (3 ml), containing 50 mM potassium phosphate buffer (pH 7.8), 13 mM methionine, 75  $\mu$ M NBT, 0.1 mM EDTA, 2  $\mu$ M riboflavin and 50  $\mu$ l supernatant (protein extract) was prepared in the dark. Samples were exposed to 15 min of light and darkness, respectively. The absorbance was measured at 560 nm. One unit of SOD is defined as the amount of



**Fig. 8.** Catalase activity (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb[III] or Sb[V] in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

enzyme that results in 50% inhibition of the rate of nitro blue tetrazolium (NBT) reduction.

## 2.8. Carbohydrates

### 2.8.1. Carbohydrate extraction

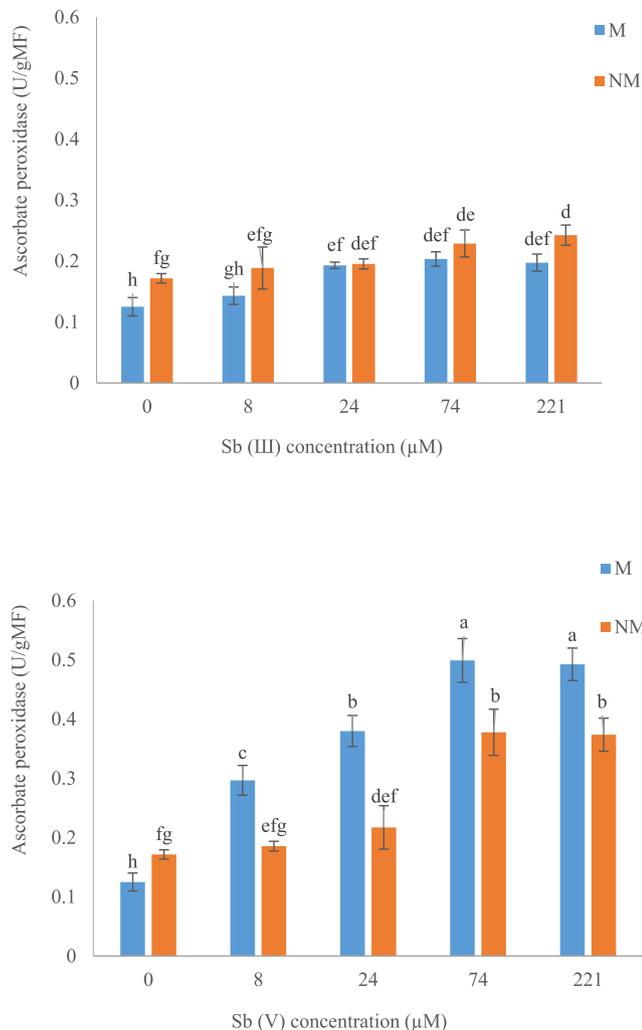
Dry leaf tissue (10 mg) was homogenized in 10 ml of warm distilled water and filtered through Whatman No.1 filter paper. The extract was used for the determination of soluble and reducing carbohydrates.

### 2.8.2. Total Soluble Carbohydrates (TSC)

Soluble carbohydrates were assayed according to Dubois et al. (1956). One ml of 80% phenol, followed by 5 ml of concentrated sulfuric acid were added to 2 ml of carbohydrate extract, and left at room temperature for 10 min. The samples were then incubated at 20–30  $^{\circ}C$  for 15 min for color development, and the absorption at 490 nm was measured. To make the standard curve, pure glucose was used at concentrations of 0, 5, 10, 20, 40, 80 and 160  $\mu$ g/ml.

### 2.8.3. Total Reducing Sugars (TRS)

Reducing sugars were determined using the dinitrosalicylic assay (DNS) (Jeffries et al., 1998). Two ml of carbohydrate extract was added to 1 ml of DNS reagent. Samples were incubated at 100  $^{\circ}C$  in a water bath for 10 min, and mixed with 10 ml of distilled water. The absorbance was measured at 546 nm and calibrated using pure glucose as a standard (see above).



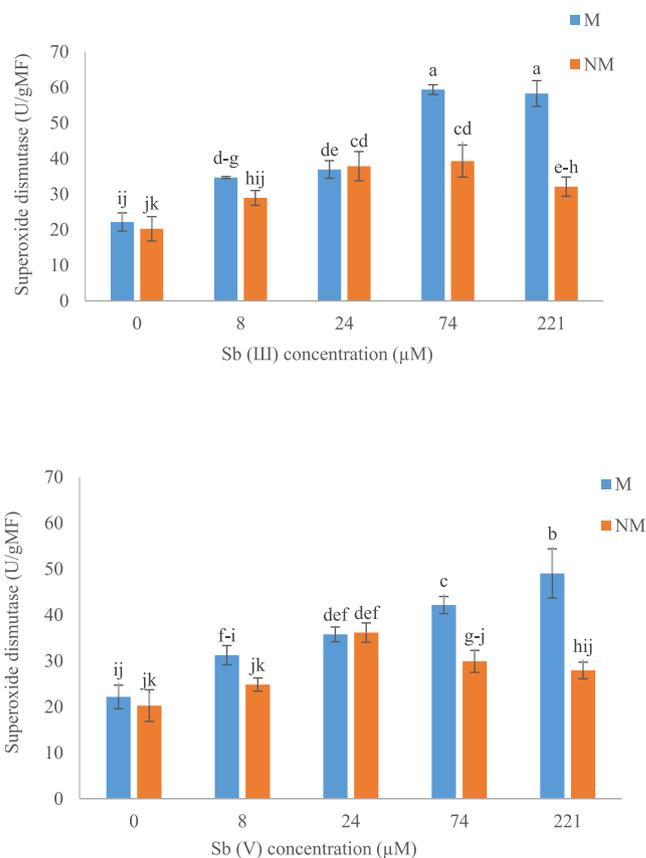
**Fig. 9.** Ascorbate peroxidase activity (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb[III] or Sb[V] in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

### 2.9. Statistics

Results were statistically analyzed using two-way ANOVA. A posteriori comparison of individual means was performed using Tukey's test (Sokal and Rohlf, 1981). To calculate the Sb redox status  $\times$  population interaction, the data from the 24-, 74-, and 221- $\mu$ M treatment levels were expressed as % of the mean control and analyzed using two-way ANOVA with Sb redox status (Sb[III] versus Sb[V]) and population as main factors.

### 3. Results

The contents of the chlorophylls *a* and *b* and total chlorophyll concentration were significantly affected by both Sb[III] and Sb[V] in both populations. Both for Sb[III] and Sb[V], the population\*Sb concentration interactions were consistently significant (Table 1). The Sb redox status\*population interaction was not significant for chlorophyll *b* concentration, but significant for chlorophyll *a* concentration ( $P < 0.05$ ) and close to significant for total chlorophyll concentration ( $P < 0.1$ ), probably because chlorophyll *a* concentration, in contrast to chlorophyll *b* concentration, was significantly and strongly enhanced by lower concentrations of Sb[V], but much less by Sb[III] in the M population, but not in the NM one, in which both chlorophylls tended to



**Fig. 10.** Superoxide dismutase activity (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb[III] or Sb[V] in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

decrease with both Sb[III] and Sb[V] (Figs. 1–3).

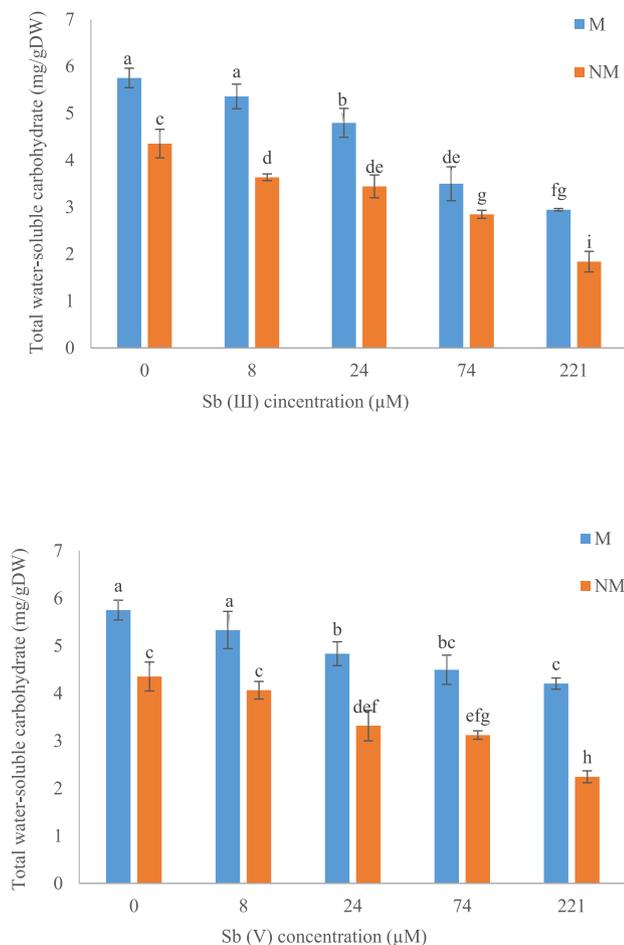
Anthocyanin concentrations were overall significantly enhanced by both Sb[III] and Sb[V] in both populations, except at the highest exposure level in the NM population (Fig. 4; Table 1), and the population\*Sb concentration interactions were significant, both for Sb[III] and Sb[V] (Table 1). The Sb redox status\*population interaction was not significant (Table 1).

The proline concentrations were significantly affected by both Sb [III] and Sb[V] (Table 1). The population\*Sb concentration interactions were significant, both for Sb[III] and Sb[V] (Table 1), apparently because proline accumulation was strongly and significantly induced by both Sb[III] and Sb[V] in M, but barely (Sb[III]), or not at all (Sb[V]), in NM (Fig. 5). The Sb redox status\*population interaction was close to significant (Table 1), apparently because Sb[V] induced much more proline accumulation than Sb[III] did in M, but barely or not in NM (Fig. 5).

The phenolics and flavonoids content were significantly enhanced by both Sb[III] and Sb[V] in both populations, except at the highest exposure levels, with significant Sb[III]\*population and Sb [V]\*population interactions (Table 1, Fig. 6, Fig. 7). The Sb redox status\*population interactions were not significant (Table 1).

Catalase (CAT) activity was significantly enhanced by both Sb[III] and Sb[V] in both populations, with significant Sb[III]\*population and Sb[V]\*population interactions (Fig. 8, Table 1). Also the Sb redox status\*population interaction was significant (Table 1), apparently because Sb[III] more strongly induced CAT activity in M than it did in NM, whereas Sb[V] induced CAT activity to a similar degree in M and NM (Fig. 8).

Ascorbate peroxidase (APX) activity was significantly enhanced,



**Fig. 11.** Total water-soluble carbohydrates content (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb[III] or Sb[V] in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

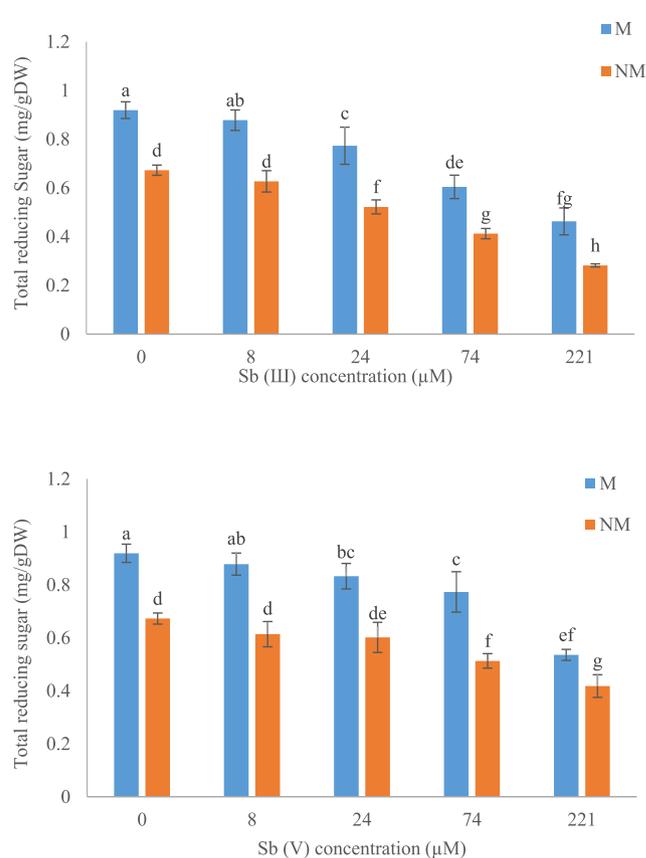
both by Sb[III] and, to a higher degree, Sb[V], in both populations, with significant Sb\*population interactions, particularly for Sb[V] (Table 1, Fig. 9). The Sb redox status\*population interaction was highly significant, apparently because Sb[V] induced much more APX activity in M than it did in NM, whereas the Sb[III]-induced APX activities were much less different (Fig. 9).

Superoxide dismutase (SOD) activity was significantly enhanced by both Sb[III] and Sb[V], but more strongly in M than in NM, resulting in significant population\*Sb concentration interactions (Table 1, Fig. 10). The Sb redox status\*population interaction was not significant (Table 1), apparently because the differences between the populations in the Sb[III] and Sb[V] treatments were very similar (Fig. 10).

The total soluble carbohydrates (TSC) and total reducing sugars (TRS) concentrations were significantly decreased by both Sb[III] and Sb[V], to a similar degree in both populations, resulting in insignificant population\*Sb concentration interactions. Also the Sb redox status\*population interactions were not significant (Table 1, Fig. 11, Fig. 12).

#### 4. Discussion

In a previous study it has been demonstrated that the M population is hypertolerant to Sb, particularly to Sb[V], but to a lower degree also to Sb[III], although the latter may, in view of the rapid oxidation of Sb [III] in aerated nutrient solutions, in fact reflect Sb[V] hypertolerance, or represent a pleiotropic by-product of As hypertolerance (Jamali



**Fig. 12.** Total reducing sugars content (mean  $\pm$  SE) in *S. spinosa* after 20 days at different concentrations of Sb[III] or Sb[V] in the nutrient solution. Different letters indicate significant differences ( $P < 0.05$ ) between means.

Hajiani et al., 2017; Rajabpoor et al., 2019). In any case, the effects of the Sb[III] and Sb[V] treatments on the contents of total chlorophylls and chlorophyll *a* concentration are fully in proportion with the corresponding effects on biomass productivity (Rajabpoor et al., 2019), showing that the apparent differences between the populations in their tolerance to the Sb[V] and Sb[III] treatments are in a proportional way reflected by the chlorophyll contents observed in the present study. In particular, the significance of the Sb redox status\*population interactions for chlorophyll *a* and total chlorophylls concentration is in line with the hypothesis that the M population is primarily hypertolerant to Sb[V] (Rajabpoor et al., 2019), and possibly, at most to a much lower degree, also to Sb[III].

Concerning the hypothesis that non-enzymatic antioxidants or osmoprotectants could be involved in Sb[V] hypertolerance, it seems that phenolics and flavonoids are not among the plausible candidates, in particular because their concentrations are similarly enhanced by Sb [III] and Sb[V], or even more strongly by Sb[III], and that the Sb redox status\*population interactions are far from significant. Of course, it remains well possible that these compounds are involved in the 'constitutive Sb tolerance' machinery, which is expected to be present in both populations. However, the pattern observed for proline accumulation, i.e. more strongly induced by Sb[V] than by Sb[III] and more in M than in NM, with an almost significant Sb redox status\*population interaction, is in fact compatible with a function in Sb[V] hypertolerance. The underlying mechanism is not evident, however. It is known that Sb exposure can lead to ROS accumulation (Ortega et al., 2017), which is confirmed by the massive upregulation of many antioxidants observed the present study, and it has been claimed that proline could act as a ROS scavenger (Signorelli et al., 2014), although it is very

difficult to imagine a plausible chemical mechanism. More likely, since toxic metal exposure generally causes drought stress (Barcelo and Poschenrieder, 1990), proline might act as an osmoprotectant, preventing cytoplasmic dehydration, or dehydration-induced protein denaturation (Verbruggen and Hermans, 2008). Of course, it is also possible that the observed coincidence of enhanced proline accumulation and Sb hypertolerance is merely accidental. Further research is needed to elucidate the precise role of proline, if any, in Sb hypertolerance.

Concerning the potential role for enzymatic antioxidants in Sb hypertolerance, it seems that CAT and SOD can be ruled out. Catalase activity did show a highly significant Sb redox status\*population interaction, and was more induced in M than in NM, but the latter exclusively in the Sb[III] treatment. In fact, the observed pattern of CAT activity is compatible with the hypothesis that CAT is a 'stress marker' with a slightly lower threshold induction level in M than in NM. Of course, this does not mean that CAT would not play any role in the constitutive Sb tolerance machinery in both populations (see above). The same also seems to apply to SOD activity, which is more strongly induced in M than in NM, but to a comparable degree by Sb[III] and Sb[V]. On the other hand, APX activity was more strongly induced in M than in NM, but exclusively by Sb[V], resulting in a highly significant Sb redox status\*population interaction. Therefore, APX induction cannot be ruled out as a potential Sb[V] hypertolerance mechanism. On the other hand, metal hypertolerance mechanisms are usually constitutively expressed, independent of exposure to the metal(s) in question (Li et al., 2017), while the M population showed an even lower APX activity than NM did under control conditions. Since completely inducible metal hypertolerance mechanisms have never been reported thus far, as to our knowledge, it seems unlikely that APX expression would represent a major determinant of Sb[V] hypertolerance, but its precise role, if any, should be addressed in future research. Of course, the same argument is also applicable to proline accumulation.

It is remarkable that the obvious hypertolerance of the M population to at least Sb[V] is apparently not reflected by the concentrations of total water-soluble carbohydrates or reducing sugars, for which both Sb\*population interactions were insignificant. We have no explanation for this phenomenon, but it may be taken to indicate that the total and reducing sugar concentrations are not directly dependent on the photosynthetic capacity, at least not in the prevailing experimental settings. Also anthocyanins did apparently not reflect differential tolerance, specifically to Sb[V], between M and NM, in view of the insignificance of the Sb redox status\*population interaction.

## 5. Conclusions

Among an array of stress markers, enzymatic antioxidants, non-enzymatic antioxidants, or osmoprotectants with a potential role in the 'constitutive tolerance' or (largely Sb[V]-specific) 'hypertolerance' to Sb in *S. spinosa*, only the chlorophyll *a* and total chlorophyll concentration, proline accumulation, and the CAT and APX activities showed a significant, or close to significant Sb redox status\*population interaction. Chlorophyll *a* concentration and, to a lower degree, CAT activity seemed to reflect the level of toxicity stress, both in the M and NM populations. APX activity and proline accumulation were more strongly induced by Sb[V] than by Sb[III], particularly in the M population, and are, therefore, potential candidates for a role in Sb[V] hypertolerance. The other compounds and enzymes (chlorophyll *b*, anthocyanins, total soluble sugars, reducing sugars, phenolics, flavonoids, catalase, superoxide dismutase) are almost certainly not involved in Sb[V] hypertolerance, but many of them might well contribute to the constitutive Sb [III] or Sb[V] tolerance in both populations.

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