



Oxidative and antioxidative responses to antimony stress by endophytic fungus *Aspergillus tubingensis* isolated from antimony accumulator *Hedysarum pallidum* Desf.

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Abstract

Antimony (Sb) is a toxic metalloid whose pollution has become a serious problem. However, studies on fungal endophytes resistant to antimony are virtually nonexistent. An endophytic fungal strain was isolated for the first time from the roots of *Hedysarum pallidum* Desf. which is a Sb accumulator *Fabacea* growing on mine cuttings. Experiments with high Sb increased concentrations (0, 5, 10, 20 and 30 mM Sb) were performed in order to assess the strain potential in contaminated environments bioremediation and to understand its Sb tolerance strategy. The isolated strain was identified as *Aspergillus tubingensis* MH189391 by morphological characteristics and phylogenetic analysis. It exhibited a minimum inhibitory concentration (MIC) of 500 mM Sb, i.e. 60,880 mg L⁻¹, and maintained high amounts of biomass up to 30 mM Sb, i.e. 3652.8 mg L⁻¹ of Sb. A stimulation of *A. tubingensis* growth and its antioxidant responses was observed at the level of 5 mM Sb, i.e. 609 mg L⁻¹. Hydrogen peroxide (H₂O₂) and malondialdehyde (MDA) contents increased significantly ($p < 0.05$) with Sb treatments. Oxidative stress induced significant increases ($p < 0.05$) in antioxidant biomarkers such as proline, catalase (CAT), and superoxide dismutase (SOD), but it resulted in a significant decrease of peroxidase (POD) and ascorbate peroxidase (APX) activities. Proline, CAT, SOD, H₂O₂ and MDA were significantly ($p < 0.05$) and positively correlated, which highlights their coactions in oxidative stress fighting. Results indicate that *Aspergillus tubingensis* has developed an important adaptation to excessive Sb concentrations and that it could be used in antimony-contaminated environments bioremediation.

Keywords Metalloid · Antioxidants · Endophyte · Fungus · Mine cuttings

Abbreviations

APX Ascorbate peroxidase
CAT Catalase
MDA Malondialdehyde
MIC Minimum inhibitory concentration

p Probability value
PCR Polymerase chain reaction
POD Peroxidase
r Correlation coefficient
ROS Reactive oxygen species
SEM Standard error of the mean
Sb Antimony
SOD Superoxide dismutase

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Introduction

Antimony (Sb) is a toxic metalloid existing in trace amounts in uncontaminated soils where it ranges in average from 0.3 mg kg⁻¹ to 8.4 mg kg⁻¹ (Clemente 2013). Sb was considered as a priority Pollutant by the United States Environmental Protection Agency (Zhou et al. 2018). A few decades ago, Sb pollution was relatively localized and concerned mining areas

and the surroundings of some industries mainly. But in the last 30 years, this metalloid has become a widespread pollutant. In fact, according to Mubarak et al. (2015), soil contamination by antimony due to human activities has considerably increased in the recent past. Sb uses by man are numerous and very diverse. The main use of Sb is as a hardener for lead in lead-acid batteries, cable sheaths and ammunition and as an important component in semiconductors. Large amounts of Sb (such as antimony trioxide Sb_2O_3) are used as flame retardants in textiles, paper, plastics and adhesives without any prospects for the development of alternative materials. Sb is also used in ceramics as an opacifier, in paints as a mordant, in brake linings, in pest control agents, in polyethylene terephthalate (PET) plastics and as an additive in the tire vulcanization process (Clemente 2013; Pierart et al. 2015).

Antimony pollution increase may constitute a serious threat to human health and to other living organisms. The development of Sb removal biotechnologies of contaminated sites is therefore necessary. Bioremediation, in situ treatment, provides a safe and economic alternative to the physicochemical strategies commonly used (Ma et al. 2016). Metal accumulating plants harbor in their roots microorganisms, known as endophytes, with potential to accumulate metals from polluted environment and to enhance metal uptake by plants (Ma et al. 2016). Thus, such endophytes, or both endophytes and their hosts, could be used in metalloid polluted environments bioremediation. In the host-endophytic association, host tolerance to abiotic stress is enhanced by an increase in antioxidant activity by the symbiont whose antioxidants production is also increased (Devi et al. 2017). Such an increase in antioxidants by the endophyte is a sign of its resistance to the pro-oxidants involved. Thus, the measurement of the antioxidants produced by an endophyte exposed to a metal stress would highlight its degree of resistance to the metal involved and therefore its possible use in the bioremediation of metal contaminated environments. However, the antioxidant system role in fungi resistance towards this metalloid remains virtually nonexistent.

In the Djebel Hamimat area (southeast of Constantine, Algeria) an abandoned mining exploitation has generated important quantities of soils containing various toxic metals, mainly antimony whose concentration reached $81.446 \text{ mg kg}^{-1}$ (Benhamdi et al. 2014). In this area, a chemical analysis of a metallophyte, *Hedysarum pallidum* Desf., carried out by the authors in question highlighted up to 263 mg kg^{-1} of Sb in its aerial parts and 183 mg kg^{-1} in its underground parts. The above authors showed that this species exhibited significant antioxidant enzymatic activities in its two parts to fight oxidative stress generated by metallic pollution. Thus, it is possible that this plant harbors endophytic fungi in its roots that would help it to tolerate high levels of this metalloid in its tissues. Such endophytes could be resistant to antimony high levels and present a powerful antioxidant

defense system that make them potential candidates for bioremediation of antimony-contaminated environments.

The purpose of the present work is to highlight and identify the most antimony tolerant endophytic fungus from *H. pallidum* roots by determining its MIC. The goal is also to determine the strain tolerance strategy to Sb by studying the metalloid impact on the antioxidant biomolecules induction. Results would determine the strain's ability to bioremediation of contaminated environments by this metalloid.

Materials and methods

Site characteristics and plant sampling

The study area is located 90 km away from Constantine, more precisely in the province of Oum El Bouaghi, a semiarid region in the northeast of Algeria with the following geographical coordinates: $35^{\circ}58'37.64'' \text{ N } -7^{\circ}11'22.80'' \text{ E}$ to $36^{\circ}01'09.78'' \text{ N } -7^{\circ}14'38.38'' \text{ E}$ (Benhamdi et al. 2014). In this area, an old abandoned antimony surface mine is situated on the side of a mountain (Djebel Hamimat) with an average altitude of 865 m (Bentellis et al. 2014).

Soils and plant roots were taken from the most contaminated spoil of the study area. The soils showed the following physicochemical characteristics: $62,000 \text{ mg kg}^{-1} \pm 4,924 \text{ Sb}$, $2,300 \text{ mg kg}^{-1} \pm 186 \text{ As}$, $0.9 \pm 0.04\%$ Organic matter (OM), $\text{pH } 8.01 \pm 0.09$, $7.8 \pm 1.82\% \text{ CaCO}_3$, $23.0 \pm 4.9\%$ Clay, $16.3 \pm 1.02\%$ Silt, $53.7 \pm 3.22\%$ Sand (Benhamdi et al. 2014).

Eight specimens of *H. pallidum* were randomly chosen from the analyzed spoil. The roots of each specimen were extracted with sterile auger, taken with sterile gloves and put in sterile flasks after being separated from the aerial part by sterile scissors.

Isolation and selection of endophytes resistant to antimony

In order to isolate fungal endophytes, *H. pallidum* roots were washed with sterile water to remove soil particles, then immersed successively in 75% (v:v) ethanol solution for 2 min and in sodium hypochlorite (35° Chlorine) at 5% for 1 min, finally, washed in sterile water three times to remove the surface sterilization agents. After the last rinse and to check the effectiveness of the disinfection, 0.3 mL of the final rinse water were inoculated into the potato dextrose agar (PDA) medium supplemented with gentamicin and incubated at 30 °C. The absence of any fungal growth on this medium indicated that all the fungi that have been isolated are endophytes (Deng et al. 2011). After that, the washed roots were cut into small pieces, placed on PDA medium and incubated at 30 °C for 1 to 2 weeks. After incubation, the obtained strains were transplanted several times on PDA

medium in order to be purified. The purified strains were stored at 4 °C (Deng et al. 2011).

To screen the antimony resistant fungal endophytes, the obtained isolates were grown on Sabouraud agar containing per liter of medium: 10 g peptone, 20 g glucose, 20 g agar, and increasing amounts of antimony in the form of tartrate ($C_8H_4K_2O_{12}Sb_2 \cdot 3H_2O$ from Sigma-Aldrich). The pH of the medium was maintained at 6.4 before autoclaving (Mukherjee et al. 2010). The selection cultures were incubated at 30 °C for 96 h. The fungal growth was monitored daily by reference to the control (Sabouraud agar culture without addition of metal). The lowest concentration of the metalloid, which completely inhibits the growth of the isolates, was considered as the minimal inhibitory concentration (MIC).

Resistant strain identification

The resistant isolate was identified by the cultural, morphological characteristics and analysis of the sequences of the calmodulin gene, TUB1/TUB2 (5' TCGACAGCTATTTC CCCCTT 3') / (5' TAGCATGTCATATCACGGGCAT 3') by PCR amplification of specific primers.

The sequences obtained after sequencing at Eurofins/Genomics Laboratory (Germany) were subjected to a sequence homology search using the BLAST program at the National Center for Biotechnology Information (NCBI) to search for the closest sequences deposited in Genbank (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). For this purpose, the sequences of the calmodulin gene were aligned with the sequences of the similar fungi retrieved from the databases using CLUSTAL X, and a phylogenetic tree was constructed using the neighbor-joining method (MEGA version 7).

Preparation of fungal biomass for the determination of Sb toxicity biomarkers

The biomass of the resistant strain was obtained from submerged cultures in the modified Sabouraud broth, composed of 50 mM citrate buffer (pH 6.4): 10 g peptone and 20 g glucose, per liter.

The total volume, 50 mL of buffered Sabouraud broth, was transferred to 250 mL Erlenmeyer flasks. The broth medium was inoculated with spores of the resistant strain and then incubated at 30 °C for 96 h in a rotary incubator at 175 rpm (New Brunswick Scientific, Co., Inc., NJ, USA).

For the biomarkers determination, four media were prepared, aseptically and separately, by supplementing Sabouraud broth with Sb increasing amounts (5, 10, 20 and 30 mM) in tartrate form. A control medium was prepared with buffered Sabouraud broth without any Sb additions. The maximum dose was determined by the minimum amount of biomass necessary to perform the biomarker toxicity analysis. The biomass was recovered after a 96 h incubation, after

filtration and rinsing with sterile bi-distilled water. It was then stored at –20 °C until use (Mukherjee et al. 2010).

The pH variations before inoculation and after obtaining the biomass were stabilized by the addition of citrate buffer to the culture media according to the method of Chakraborty et al. (2014).

Intracellular H₂O₂ assay

The intracellular H₂O₂ content was measured according to the method described by Chakraborty et al. (2014). The fresh mycelia (0.5 g) were homogenized in an ice bath with 5 mL of 0.1% TCA (w:v). The homogenate was centrifuged at 12000×g for 15 min, then 0.5 mL of the supernatant was added to 10 mL of the phosphate buffer (0.5 mM, pH 7.0) and 1 mL of the 1 M KI. The absorbance was read at 390 nm. The H₂O₂ contents were determined from a standard curve.

Malondialdehyde (MDA) assay

The MDA was assayed following the method used by Benhamdi et al. (2014) in order to evaluate the level of lipid peroxidation. Fresh biomass (0.5 g) was crushed in liquid nitrogen and homogenized with a 5 mL solution containing 20% trichloroacetic acid (TCA) and 0.5% thiobarbituric acid (TBA). The mixture was incubated at 95 °C for 30 min and the reaction was stopped by immersing the tubes in ice. The cooled mixture was centrifuged at 5000×g for 10 min at 25 °C. The non-specific absorbance at 600 nm was subtracted from the absorbance at 532 nm and the MDA concentration was determined by its molar extinction coefficient: 155 mM⁻¹ cm⁻¹. It was expressed in μM g⁻¹ of fresh biomass.

Intracellular proline assay

The intracellular proline content was determined by the method adopted by Zhang et al. (2011). Fresh biomass (0.1 g) was ground in an ice bath. Then, 2 mL of 40% methanol (v:v) were added after incubation at 85 °C for 60 min and cooled to room temperature. After decantation, 1 mL of the supernatant was mixed with 2 mL of acetic acid, 25 mg ninhydrin and 1 mL of a mixture containing: acetic acid, distilled water and orthophosphoric acid (120:300:80; v:v:v). The mixture was boiled for 30 min and then placed in an ice bath to stop its reaction. In order to allow the phase separation, toluene was added to the mixture and stirred vigorously. The intensity of the red color of the upper phase was read at 528 nm. The concentration of proline was determined using the extinction coefficient 0.62 μM⁻¹ cm⁻¹ relative to the fresh weight.

Antioxidant enzyme activities assay

For antioxidant enzyme activities measures, the fresh biomass of the resistant fungal strain (0.5 g) was ground into a fine powder with a mortar in liquid nitrogen. The proteins were then extracted at 4 °C with 3 mL of cold potassium phosphate buffer (50 mM, pH 7.0) containing 0.1% triton X-100 (v:v) and 1% polyvinylpyrrolidone (PVP) (w:v). The homogenate was centrifuged at 14000×g at 4 °C for 15 min and the supernatant was used to measure antioxidant activities (Mukherjee et al. 2010).

Protein concentrations were quantified according to Lowry et al. (1951) method, using BSA (*bovine serum albumin*) as a standard.

The activity of catalase (CAT) (EC.1.11.1.6) was measured according to the method of Chance and Maehly (1955). The decomposition of hydrogen peroxide was determined by its absorbance decrease at 240 nm ($\epsilon = 39.4 \text{ mM}^{-1} \text{ cm}^{-1}$). The activity of CAT was expressed in $\mu\text{M min}^{-1} \text{ mg}^{-1}$ proteins.

The total activity of SOD (EC.1.15.1.1) was evaluated according to the method of Marklund and Marklund (1974) by its ability to inhibit autoxidation of pyrogallol at 420 nm. The SOD unit was defined as the amount of enzyme required to cause 50% inhibition of pyrogallol autoxidation.

The POD activity (EC.1.11.1.7) was quantified according to the method of Chance and Maehly (1955); the amount of purpurogallin formed between H_2O_2 and pyrogallol was measured at 420 nm and the enzymatic activity was expressed as $\text{U} \cdot \text{min}^{-1} \text{ mg}^{-1}$ proteins.

The activity of APX (EC.1.11.1.11) was measured according to the method of Nakano and Asada (1980) by decreasing in the absorbance of oxidized ascorbate in the presence of H_2O_2 at 290 nm ($\epsilon = 2.8 \text{ mM}^{-1} \text{ cm}^{-1}$). The enzymatic activity was expressed in $\mu\text{M min}^{-1} \text{ mg}^{-1}$ proteins.

Statistical analysis

The obtained data for the different tested biomarkers at increased Sb concentrations were presented in bar graphs and correspond to means (\pm SEM) of 3 repetitions. The significance of the differences between the means, at the level of $p < 0.05$, was estimated using a variance analysis (ANOVA) followed by the Tukey's test. The relationships between all analyzed variables and between those and Sb concentrations of the medium were tested by the Pearson correlation coefficients (r). The data treatment was carried out using XLStat software (XLStat 2014, Addinsoft, Paris, France).

Results

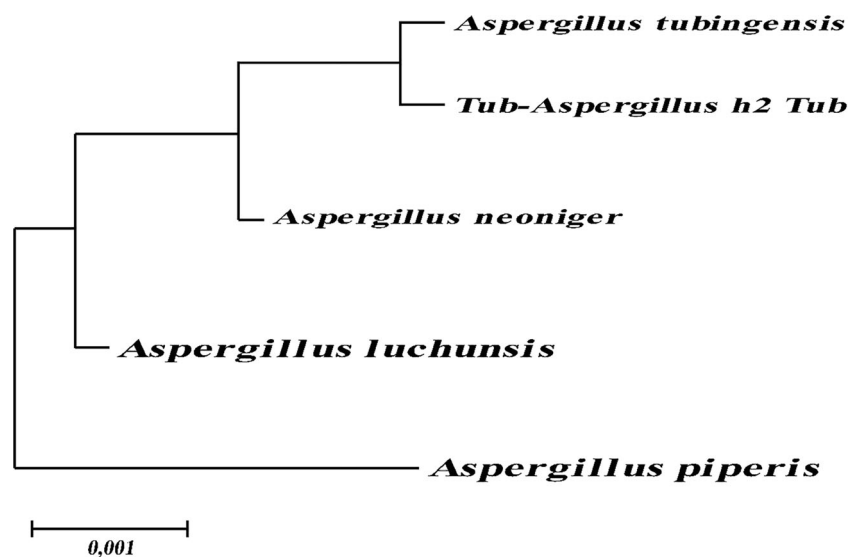
Antimony-resistant strain identification

Of the 36 endophytic strains isolated from the roots of *Hedysarum pallidum* Desf., only the isolate Tub-H2 was able to grow up to 500 mM Sb. This strain showed a rapid growth at 28–30 °C by covering the agar without Sb with a white powder at the beginning of the growth which became black during the development. The mycelium has distinct septa and black brown spores. The morphological and cultural characteristics of this isolate allowed its affiliation to the genus *Aspergillus*.

The analysis of the calmodulin gene sequences indicated that the resistant strain Tub-H2 was affiliated to *Aspergillus tubingensis* with a 100% similarity (Fig. 1).

Thus, the morphological and phylogenetic characteristic identification makes it possible to conclude that the resistant strain is *Aspergillus tubingensis* MH189391.

Fig. 1 Phylogenetic tree according to distance method (Neighbor-Joining) based on the analysis of calmodulin gene sequences using Mega (Molecular Evolutionary Genetic Analysis) software showing the phylogenetic position of the Tub-H2 isolate



Antimony effect on fungal biomass

Biomass of *Aspergillus tubingensis*, represented by its dry weight (Fig. 2a), decreased significantly with the increase of Sb concentrations. It was very significantly and negatively correlated with the metalloid concentrations (Table 1). At 5 mM Sb, *A. tubingensis* biomass increased significantly by 25% compared to the control and was

significantly higher than the biomass obtained with all the Sb concentrations.

Intracellular H₂O₂ contents

The intracellular H₂O₂ contents of *A. tubingensis* (Fig. 2b) did not present significant increases at 5, 10 and 20 mM Sb, compared to the control. At 5 mM Sb, they were

Fig. 2 Dry weight and Biochemical responses measures, according to Sb concentrations, in the endophytic fungus *Aspergillus tubingensis* isolated from roots of *Hedysarum pallidum* Desf. **a** dry weight, **b** H₂O₂ contents, **c** MDA contents, **d** Proline contents, **e** CAT activities, **f** SOD activities, **g** POD activities, **h** APX activities. Values represent means ± SEM (N = 3). Different letters discriminate statistical differences between the measured parameters at the different antimony (Sb) concentrations (using ANOVA followed by Tukey’s test, p < 0.05)

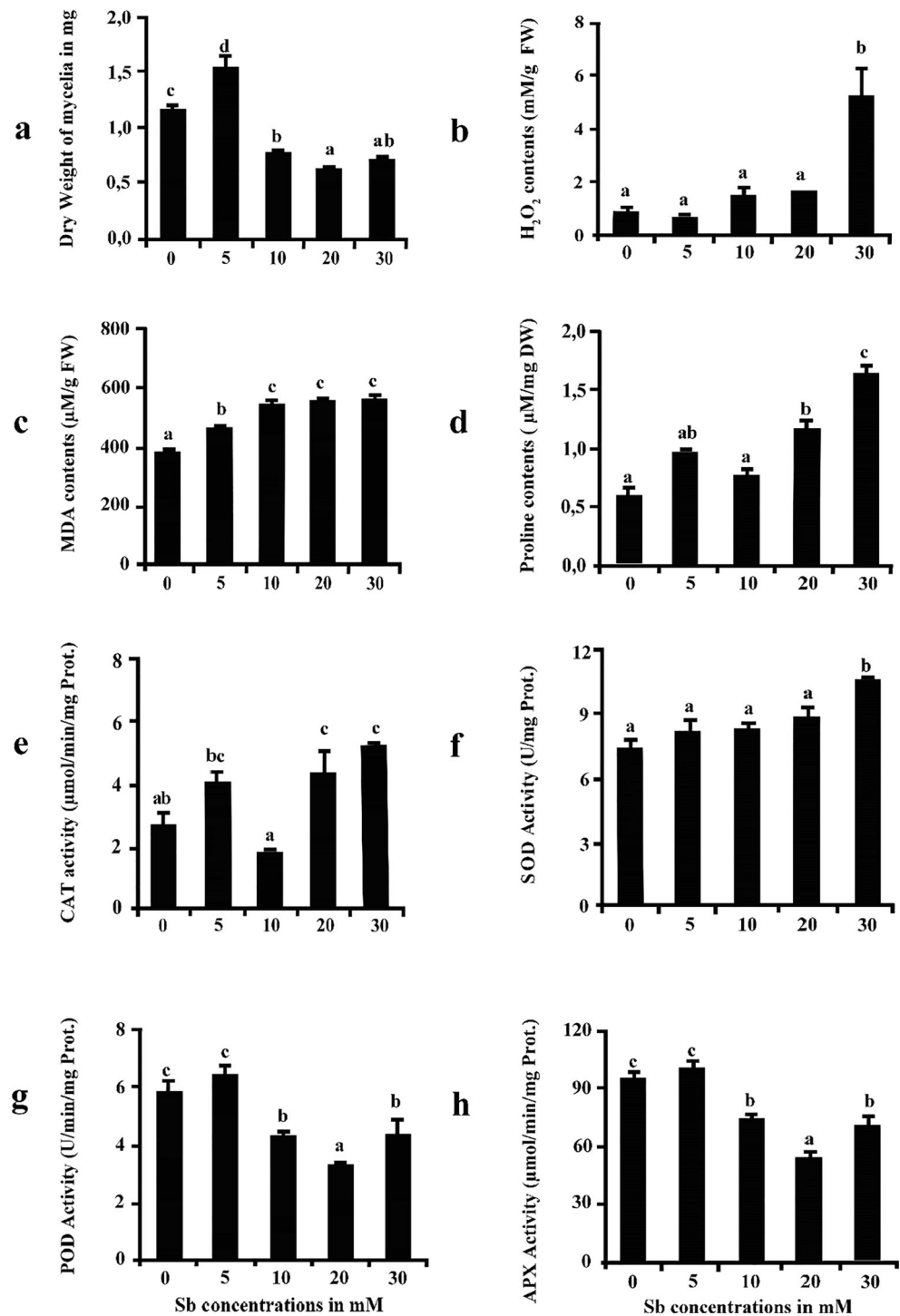


Table 1 Pearson correlation coefficients between all analyzed variables and between those and antimony culture medium concentrations

	Dry weight	H ₂ O ₂	MDA	Proline	CAT	SOD	POD	APX
Sb (mM)	−0,710**	0,868***	0,841***	0,934***	0,688**	0,919***	−0,656**	−0,745**
Dry weight	1	−0,535*	−0,706**	−0,436	−0,096	−0,498*	0,927***	0,911***
H ₂ O ₂		1	0,611*	0,858***	0,605*	0,883***	−0,424	−0,456
MDA			1	0,704**	0,372	0,711**	−0,712**	−0,800**
Proline				1	0,850***	0,711**	−0,395	−0,511*
CAT					1	0,755**	−0,152	−0,277
SOD						1	−0,451	−0,525*
POD							1	0,932***

* $p < 0.05$; ** $p < 10^{-2}$, *** $p < 10^{-3}$ ($n = 15$)

slightly lower than those of the control, whereas at 10 and 20 mM Sb they showed an increase of 174% and 197% respectively, compared to the control. At 30 mM Sb, the intracellular H₂O₂ contents were significantly higher than the control that they exceeded by 631%. They were very significantly and positively correlated with Sb concentrations in media but negatively and significantly with strain's dry weight (Table 1).

MDA contents

The MDA contents of *A. tubingensis* (Fig. 2c) increased significantly at 5 and 10 mM Sb compared to the control. At 5 mM Sb they exceeded those of the control only by 30%, whereas at 10 mM Sb they exceeded it by 44%. From 10 to 30 mM Sb, MDA contents of the strain remained significantly invariable. They showed positive and very significant correlation with media Sb concentrations, significant positive correlation with strain's H₂O₂, but significant negative correlation with the strain dry weight (Table 1).

Intracellular proline contents

The proline contents of *A. tubingensis* (Fig. 2d) increased significantly at 5 mM Sb. They were 69% higher than that of the control, and significantly closer to the contents obtained at 20 mM Sb level, while at 10 mM Sb, they decreased to be also closer to the control, remaining 28% higher.

The proline levels increased significantly at 20 and 30 mM Sb, exceeding those of the control by 97.50% and 177% respectively. In general, the appearance of the diagram showed a progressive increase in proline levels with increasing Sb, except at 5 mM Sb where a significant increase in proline contents was observed compared to 0 and 10 mM. Moreover, they were very significantly and positively correlated with media Sb concentrations strain's H₂O₂ and MDA contents, but

negatively correlated, although not significantly, with strain's dry weight (Table 1).

Antioxidant enzyme activities

Overall, it appears (Fig. 2e) that CAT activities increased progressively and significantly according to Sb concentrations in culture media except at 5 mM Sb. At this concentration, as for proline level, the CAT was significantly higher than that measured at 0 and 10 mM Sb and was close to activities measured at 20 and 30 mM Sb. These latter exceeded that of the control by 163% and 197% respectively. CAT activities showed positive and significant to very significant correlations with Sb concentrations, with strain's H₂O₂ and proline contents. They also showed positive correlation, although not significant, with MDA contents, and no correlation with strain's dry weight (Table 1).

SOD activities of *A. tubingensis* increased steadily, but not significantly, from 0 to 20 mM Sb (Fig. 2f). It was only at 30 mM Sb that the SOD presented an activity significantly higher than the control. However, SOD activities showed very significant positive correlations with Sb concentrations, H₂O₂, MDA, proline contents and CAT activities, but, significant negative correlations with strain's dry weight and APX activities. They also showed almost significant negative correlation with POD activities (Table 1).

The diagrams of POD and APX activities showed a very similar general aspect. They also had a important similarity to the biomass diagram. POD and APX activities (Fig. 2g and h) revealed a slight increase of 111% and 106% respectively at 5 mM Sb. Then they showed significant decrease from 10 mM Sb, compared to the control. However, the decrease at 30 mM of Sb (i.e. 3,653 mg L⁻¹) was respectively only 22% and 29% of that the control.

Both enzymes had very significant positive correlations between them and with strain's dry weight. They exhibited significant to almost significant negative correlations with

Sb concentrations, MDA, H₂O₂, proline contents, and SOD activities (Table 1).

Discussion

Aspergillus tubingensis, isolated for the first time from the roots of a steppic antimony accumulator plant *Hedysarum pallidum* Desf., growing on Sb mine spoils, proves to be a real endophyte, because it was also isolated from the roots of another species, *Pongamia pinnata*, by Huang et al. (2010), and from tissues of Mangrove trees by Bacal and Yu (2017).

Toxicity in vitro tests showed that *A. tubingensis* was tolerant at concentrations up to 500 mM Sb, namely 60,880 mg L⁻¹, which corresponds to the maximum level of Sb determined in the mining region (Benhamdi et al. 2014). Such a resistance level does not appear to be an exceptional character of this strain since Qayyum et al. (2016) already demonstrated its resistance to heavy metals.

The toxic effect of Sb on *A. tubingensis* was highlighted by the significant decrease of its biomass according to Sb concentrations increase. It was also evidenced by the very significant negative correlation of its biomass with Sb concentrations. The biomass decrease can be explained by the physiological mechanisms modification of tested strain in response to Sb toxicity. In fact, according to Chakraborty et al. (2012, 2014), non-lethal metallic amounts induce a disturbance in the membrane permeability of the fungal cell and consequently a significant loss of essential nutrients for fungal growth. However, *A. tubingensis* still preserved a significant biomass (61% of the control) at 30 mM Sb, namely 3,653 mg L⁻¹ Sb. Such a result shows an important resistance of this strain to Sb toxicity.

The significant increase in biomass at 5 mM Sb compared to the control (0 mM Sb), instead of its decrease, shows that the strain's growth was stimulated by the presence of Sb. The concentration of 5 mM, ie 609 mg L⁻¹, is relatively high since the normal levels of Sb in soils are inferior to 8 mg kg⁻¹ (Clemente 2013). It is also much higher than the maximum Sb content (183 mg kg⁻¹) determined in the roots of *H. pallidum* from which *A. tubingensis* was isolated (Benhamdi et al. 2014). Such a result suggests that the adaptation of this fungal strain to excessive Sb levels in the natural environment has resulted in this element becoming necessary for its development at a relatively high threshold concentration. Beyond this threshold, Sb becomes toxic for the strain by reducing its growth, but its toxicity remains low since the strain still had an important biomass.

The toxicity of antimony in the medium induced the formation of reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂). This was evidenced by the very significant positive correlation of Sb concentrations with H₂O₂ contents. However, it was only from 30 mM Sb that the production of H₂O₂ became important by being significantly higher than the

control. Indeed, H₂O₂ contents produced at the lowest concentrations (5, 10 and 20 mM Sb) were not significantly different from that of the control medium (0 mM Sb). As a result, the oxidative stress generated by antimony toxicity become significant only from 30 mM Sb, indicating an important resistance of the strain to concentrations below this level, but which are still very high.

The ROS, including H₂O₂, induced by Sb treatments, have involved a lipid peroxidation (LPO) of the polyunsaturated fatty acids, of which the final product is malondialdehyde (MDA). Such a result is highlighted by the very significant positive correlation of H₂O₂ contents with MDA.

MDA is a reliable indicator of free radical formation. It is used as a biomarker of toxicity in a variety of living organisms (Tsikas 2017). It indicates membrane damage leading to a disruption of the metabolic function and a reduction of cellular integrity, thus a decrease in growth. Such an effect, in the case of *A. tubingensis*, is highlighted by the significant negative correlations of the strain dry weight with its H₂O₂ and MDA contents and with the media Sb concentrations. These results are in accordance with the work of Mukherjee et al. (2010) which showed that lipid peroxidation increased steadily with the increase of arsenate amounts in *Aspergillus niger*. Similarly, Chakraborty et al. (2014) indicated that the peroxidation of *Aspergillus foetidus* cell membrane induced by cadmium, caused damages in cell wall.

The same results were observed by Benhamdi et al. (2014), they illustrated that MDA increased with soil Sb concentrations in the roots of *H. pallidum* Desf. from which *A. tubingensis* was isolated. This finding indicates a close relationship between the host plant and its endophyte.

The low MDA contents at 5 mM Sb would have caused insignificant membrane damage at this concentration, which explain the important biomass obtained at 5 mM than at 10, 20 and 30 mM Sb. However, contrary to H₂O₂ contents, MDA contents determined at the concentrations of 5, 10 and 20 mM Sb, were significantly higher than that of the control. This could be explained by the fact that other ROS than H₂O₂ were involved in the production of MDA.

The very significant and positive correlations of proline contents of *A. tubingensis* with Sb treatments, strain's H₂O₂ and MDA contents indicate that the intracellular proline increase with Sb concentrations of the medium, H₂O₂ and MDA contents. Such an increase shows that the production of proline was triggered by the presence of the ROS generated by the metalloids toxicity. It suggests that proline is involved in H₂O₂ removal and in preventing the lipid peroxidation. Furthermore, the negative correlation of proline with fungal growth suggests that this reduction, due to Sb toxicity, would be the inducing factor of proline production. Therefore, *A. tubingensis* produces intracellular proline to struggle the metalloids toxicity, reduce its deleterious effects on the metabolism, and prevent the reduction of the fungus growth. This

indicates an important adaptation of the fungal strain to the excessive contents of Sb. This adaptation seems more important at 5 mM Sb since the strain's proline contents are higher than at 0 and 10 mM Sb. Indeed, according to Raj and Mohan (2016), high levels of intracellular proline are specific characteristics of hypertolerant heavy metal populations. They suggest that intracellular proline have a functional role in high resistance to metals. The obtained results are corroborated by the works of Chakraborty et al. (2012, 2014): when studying the behavior of *Aspergillus foetidus* according to Pb (II) and Cd toxicity, they found out that intracellular proline increased in a gradual and significant way with that of the two elements in the medium.

Very significant positive correlations of proline with CAT and SOD suggest that proline production is related to the induction of these enzymatic activities. Indeed, according to the works of Zouari et al. (2016), on date palm (*Phoenix dactylifera* L. cv Deglet Nour), proline intervenes in alleviation of cadmium stress by promoting CAT activity. Not only could this be the case for *A. tubingensis* for CAT but also for SOD.

The significant increase in CAT activity with Sb treatments and H₂O₂ contents and their very significant positive correlations show that CAT activity is triggered by H₂O₂ contents, which are induced by Sb toxicity. It suggests that CAT is involved in H₂O₂ removal. In fact, catalases are known to be implied in one of the mechanisms that protect cells against cellular components damages caused by H₂O₂ and reactive oxygen species (ROS) (Teng et al. 2018). Moreover, according to these authors, catalase activity is directly regulated by H₂O₂ concentrations in the medium. So, the H₂O₂ formed by Sb toxicity would be eliminated by CAT, produced by *A. tubingensis*, that neutralizes or reduces its toxic effects. Such a result indicates an important adaptation of the fungal strain to the presence of excess Sb in the medium. This adaptation seems more accentuated at 5 mM Sb since CAT activity is higher than at 0 and 10 mM Sb. As it was for MDA, the CAT activity of the plant *H. pallidum*, had increased significantly with the increase of antimony pollution in soil (Benhamdi et al. 2014). Such an increase could therefore be related to the presence of *A. tubingensis* in *H. pallidum* roots.

Superoxide dismutase (SOD) is another antioxidant enzyme that protects cells against oxidative stress. Thus, the increase of SOD activity in *A. tubingensis* with increased Sb treatments, which are correlated significantly and positively, prove that the metalloid induced SOD activity of the strain.

Indeed, according to Demidchik (2015), SOD reacts with superoxide radicals (O₂^{•-}) to produce H₂O₂ and O₂. The production of H₂O₂ by SOD makes it possible to reduce the amount of O₂^{•-} produced by the stress and thus to prevent the formation of hydroxyl radicals (OH[•]) in the Haber-Weiss reaction. These hydroxyl radicals are the most reactive species causing LPO, consequently, MDA production. Therefore, the

very significant positive correlations of SOD with H₂O₂ and MDA suggest that SOD is involved in H₂O₂ and MDA production, so, in LPO prevention.

The H₂O₂, which is also an oxidant, would be eliminated by intracellular proline and CAT. This, therefore, explains the very significant positive correlations found between SOD, CAT activities, H₂O₂ and proline contents.

Benhamdi et al. (2014) also highlighted an increase in SOD activity of *H. pallidum* according to Sb contamination in soils. In addition, it was more important in *H. pallidum* roots than in its aerial parts. This suggests that *A. tubingensis* could play a role in increasing SOD activity of its host.

POD and APX belong to a group of oxidoreductases found in fungi which mediate electron transfer from hydrogen peroxide (H₂O₂) and organic peroxide to various electron acceptors (Choi et al. 2014). Consequently, the similarity of POD and APX activities graphs and their very significant positive correlation could be explained by the fact that they intervene in the same type of reaction.

Significant decrease of both POD and APX activities with increased Sb concentration and the very significant and negative correlations of these enzymes with Sb treatments highlight a relative inhibition of POD and APX activities by Sb toxicity. Such inhibition could be linked to the excessive H₂O₂ production, hence explaining the negative correlations of both enzymes activities with the strain's H₂O₂ contents. Indeed, according to Demidchik (2015), peroxidases could be inactivated by their substrate, hydrogen peroxide. Similarly, Feng et al. (2016) noted a reduction in APX and POD activities of paddy rice roots exposed to antimony and selenium. The low correlation between both enzymes activities and H₂O₂ contents may be explained by the fact that other ROS, such as hydroxyl radicals, would be involved in the inactivation of *A. tubingensis*'s peroxidases.

The very significant and negative correlations between MDA contents and both enzymes suggest that they are not involved in the LPO prevention.

Since peroxidases monitor a large variety of fundamental biological processes (Demidchik 2015), their inactivation inevitably leads to reduce the growth of *A. tubingensis*. This would explain the very significant positive correlations between both enzyme activities and the strain's dry weight.

Despite their decreases, the activities of the POD and the APX are still relatively important since, at a high concentration of Sb (30 mM ie 3,652.8 mg L⁻¹), they correspond to 78% and 71% of those recorded for the control, respectively. Therefore, this attests to an important adaptation of the strain to excessive levels of antimony. Such an adaptation also appears in the exceeding of both enzyme activities at 5 mM Sb compared to those of the control and the other Sb treatments.

The plant, *H. pallidum*, from which *A. tubingensis* was isolated, exhibited, on the contrary, an increase in the activities of both enzymes according to Sb increase in soil (Benhamdi

et al. 2014). This could be explained by the fact that peroxidases of *H. pallidum* differ from those of *A. tubingensis*.

Conclusion

The fungal endophyte *Aspergillus tubingensis* isolated, for the first time, from the roots of an antimony accumulator plant, *Hedysarum pallidum* Desf., showed a high resistance to antimony by growing up to 500 mM Sb in broth media.

This strain showed an important adaptation to Sb toxicity by the establishment of antioxidant biomolecules defense systems allowing the endophyte to fight the toxicity of the medium. It appears that proline, CAT and SOD interact to eliminate H₂O₂ and avoid lipid peroxidation, while POD and APX are not involved in such a mechanism.

Similarity in some antioxidant responses of *A. tubingensis* to Sb toxicity with those of the host plant suggests that the fungal strain would participate in the protection of *H. pallidum* in trapping at least part of the ROS.

These results indicate that *A. tubingensis* developed indigenous mechanisms to tolerate high Sb doses. Although this strain was originated from terrestrial environment, its resistance to Sb was demonstrated in a liquid medium. This suggests that this strain could be a potential agent for bioremediation of both soil and aquatic environments contaminated by antimony.

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Compliance with ethical standards

Conflict of interest No potential conflict of interest or financial disclosure for all authors. All persons gave their informed consent prior to their inclusion in the study.

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