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Research Paper

Biochar-bacteria partnership improves rice growth and soil microbial community diversity while decreasing antimony accumulation and in-vitro bio-accessibility in contaminated soil

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ABSTRACT

Antimony (Sb) is a toxic metalloid impacting on plants, humans and ecosystem stability. Biochar (BC) is a promising amendment to mitigate toxic metals/metalloids. However, the role of BC and bacterial inoculation in mitigating Sb toxicity and bio-accessibility, and reshaping soil bacterial community has not yet been explored. To investigate this subject, a rice pot experiment was set up involving six treatments: unstressed soil (Ctrl); 1200 mg Sb kg^{-1} (Sb stress); Sb stress +1 % BC (1 % BC); Sb stress +2.5 % BC (2.5 % BC); Sb stress +1 % BC + *Bacillus subtilis* bio-inoculum (1 % BC + BI); Sb stress +2.5 % BC + BI (2.5 % BC + BI). The serious impairment in rice growth, physiology and final yield determined by Sb stress was reduced by BC and associated BI. The maximum stress relief was obtained with 2.5 % BC + BI, which increased rice growth and final grain yield (+85 %) by improving several plant traits and soil properties, while decreasing Sb availability. 2.5 % BC + BI curbed Sb concentration in plant organs (−43 % in the whole plant), whereas Sb whole plant content was moderately reduced (−13 %), due to a growth driven Sb uptake effect. Upon 2.5 % BC + BI, soil total Sb concentration and in vitro bio-accessibility were similarly reduced (average, −35 %) due to increases in soil total carbon (+61 %), microbial biomass carbon (+37 %), and enzymatic activities (+72 % in the average of urease and catalase). The addition of BC + BI significantly boosted the relative abundance of soil bacteria involved in reducing Sb toxicity. Our findings highlight BC + BI potential to improve rice production, reduce Sb plant accumulation, soil in-vitro bio-accessibility, and ameliorate soil bacterial community diversity.

1. Introduction

Antimony (Sb) is a toxic metalloid to plants, humans, and living organisms [1], and it is classified as a priority pollutant by the US Environmental Protection Agency and the EU Council since 1976 [2]. The maximum allowable concentration of Sb in water and soil are 20 μg L^{-1} and 36 mg kg^{-1}, respectively [1]. Nevertheless, concentration of Sb has increased to dangerous levels in some areas due to human activities. For instance, Sb concentration in ground water and soil near the Xikuangshan mines of Hunan, China has reached 47.4 mg L^{-1} and 1565 mg kg^{-1}, which are quite higher values than the allowed limits [3,4]. The concentration of Sb is increasing in waters, soils, and sediments owing to mining, smelting, weathering of sulphide ores, ammunition, and electronic materials manufacturing [5,6]. Compared to this, agricultural soils represent less serious cases of Sb pollution. Concentrations above the threshold of 36 mg Sb kg^{-1} in the soil [7] are likely to determine dangers to ecosystems and threats to human health [8].

Antimony is a non-essential metalloid, which exists in two forms:

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antimonate (Sb—V) and antimonite (Sb-III). The latter form is more reactive and easily taken up, and poses harmful impacts on all plants and living organisms [1,9]. Antimony toxicity impairs several plant functions: it disrupts principal metabolic processes, decreases antioxidant activity [10], impacts photosynthesis, enhances lipid peroxidation and oxidative stress damage [4,11–13], reduces nutrient and water uptake, in turn reducing plant growth [14,15]. A recent study conducted on rice showed that Sb inhibited the plant growth, by decreasing the photosynthesis and increasing ROS production coupled with reduced antioxidant activity [16]. Additionally, Sb alters the soil microbial population and decreases soil enzymatic activity, negatively affecting nutrient availability [17]. Antimony accumulation into edible plant parts and subsequent entry into the food chain [18] is the cause of serious health problems [1,19]. This calls for a quest to reduce plant Sb concentration, strengthen plant defenses against this stressor, and abate its occurrence in food chains.

In this perspective, different amendments, including biochar (BC), nano-materials, silicon, compost, additives, and phyto-hormones are being used across the globe to contain Sb uptake by rice plants [20,21]. Biochar is a carbon-rich material that has already shown promising results in the remediation of polluted soils owing to its porous structure and large surface area which reduce potentially toxic elements' mobility, bio-availability, and toxicity [22,23]. Biochar directly binds ions by different mechanisms, including complexation, cation exchange, electrostatic interaction, reduction, and precipitation. Biochar, especially in association with nano-particles of specific elements (Fe and Mg oxides, titanium dioxide) or compost has proved beneficial in mitigating the adverse effects of Sb, a metalloid involving a different chemical behavior with respect to metals [24–30]. However, the supply of organic matter with BC is also responsible for increased Sb bio-accessibility, potentially leading to increased Sb plant uptake [6,29,30]. In parallel to this, BC positively influences soil properties such as cation exchange capacity (CEC), soil carbon and pH, which also increase the soil retention of noxious elements [31]. Biochar has shown promising results in immobilizing the Sb and reducing its availability to plants. For instance, it was observed that modified BC decreased the soil CEC and enhanced soil catalase activity, which increased the Sb immobilization. These authors also observed that the electron attraction force between BC and Sb was increased in the oxidization-reduction process which was the fundamental reason for enhanced Sb immobilization following BC application [32]. Biochar also converts the Sb from exchangeable form to stable forms [32] and increases the immobilization of Sb by chemical reduction, electrostatic repulsion, anionic competition, and biological reduction reactions [33]. Li et al. [34] observed that modified BC enhanced Sb immobilization via iron (Fe) induced electrostatic attraction, π-π electron donor-acceptor coordination, and complexation of Sb (Fe–O(H)–/Sb). Recently, Ihenetu et al. [35] found that BC amendment decreased the Sb-III and Sb—V availability by 85–90 % via increasing dissolved organic carbon, soil fertility, microbial resilience, and stability in Sb-polluted soil. Another group of researchers observed that BC treatment decreased the soil available Sb by 14.4 % and increased the gram-negative bacteria (67.4 %) and arbuscular mycorrhizal fungi (AMF) (31.3 %). These authors also observed that BC decreased the Sb uptake and its accumulation in rice roots by increasing iron plaque formation [36]. Biochar also immobilize the Sb in soil by decreasing exchangeable forms of Sb and forming the stable forms of Sb bound to O-, Fe-, and Si-associated minerals [37]. However, Sb stabilization in soils by BC largely depends on soil properties, BC characteristics (feedstock and pyrolysis temperature), and its application rate [33].

Recently, BC, in combination with microorganisms (bacteria and fungi), has emerged as an effective measure to remediate polluted soils. This technique is effective and provides better results than the sole BC application [38,39]. Additionally, BC is an excellent carrier material for microbes, showing promising results in terms of improved plant growth and remediation of cadmium polluted soils [40]. Biochar with microbial combination provides shelter and nutrients to plants; positively influences soil properties, the microbial community, enzymatic activities, and promotes the immobilization of persistent elements, in turn reducing their availability and toxicity [41,42]. Likewise, Li et al. [43] found that Sb-oxidizing bacteria (*Bacillus* sp. S3) and Fe-modified BC increased the Sb immobilization by decreasing the bio-availability of Sb and converting it into reducible forms and improving soil properties including aggregate stability, water holding capacity and pore size distribution. They also observed that the combined use of BC and bacteria enhanced the abundance of nitrogen- and sulfur-cycling bacteria, which helped increase Sb immobilization. A recent study also showed that *Ochrobactrum oryzae* and BC mitigated the Sb toxicity and increased plant growth by increasing antioxidant activities, nutrients and carbon availability, soil enzymes activities, soil pH and abundance of favorable bacteria, while decreasing Sb availability and accumulation [44]. Another study demonstrated that coconut shell BC and bacterial partnership enhance rice productivity by decreasing Sb accumulation and improving bacterial abundance, sugars, amino acids, ketones synthesis, and purine, and glyoxylate metabolisms [45].

Rice is a fundamental component in the diet for nearly half of the world's population. However, rice plants can take up significant Sb amounts from soil [46], which is consistent with the anoxic conditions of flooded soils favoring Sb(V) reduction to Sb(III), and promoting Sb mobilization and its root uptake [8,47,48]. Antimony concentration $>$ 50 mg kg^{-1} in paddy soils can pose health risks for rice consumers [49], while this element's toxicity negatively impacts rice growth and productivity [9,50]. The application of BC alters microbial abundance and increases Sb oxidation and its immobilization, thereby decreasing its plant toxicity [47]. Biochar application also improves antioxidant performance, plant physiological functioning, and decreases the exchangeable form of Sb, which in turn mitigates the risks of Sb toxicity [32].

Overall, BC has demonstrated a promising potential but also downsides in mitigating the soil accumulation of the toxic metalloid Sb. The role of modified BC in mitigating Sb toxicity is widely studied [24–26,31]. The role of BC with bacterial inoculation in mitigating Sb toxicity, in-vitro bio-accessibility, and reshaping the soil bacterial community has not been explored yet. The present study was conducted on agricultural soil to investigate the mechanisms governing Sb mobility and bioavailability in the soil-plant system following the application of BC and bacterial inoculation which is not reported yet. We hypothesized that bacterial inoculation (BI) could more effectively decrease the Sb toxicity and remediate Sb-polluted soil than the simple BC.

This study was aimed to: (i) evaluate the efficiency of BC and BC + BI in improving rice productivity and mitigating Sb toxicity and in-vitro bio-accessibility; (ii) determine the impacts of BC and BC + BI on soil properties, Sb uptake, and partitioning to plant organs; (iii) explore the shift in soil bacteria community in Sb polluted soil following BC and BI-BC applications. The present study will provide insights to develop eco-friendly measures to remediate the Sb-polluted soils to reduce Sb uptake in crops and its subsequent entry into humans. This study increases our understanding of Sb dynamics in an agricultural setting and will provide actionable measures to mitigate Sb toxicity in plants and humans.

2. Materials and methods

2.1. Preliminary assessment of *Bacillus subtilis* 1.2172 tolerance to Sb

In order to select a suitable Sb concentration for the Sb stress treatment, potential tolerance to Sb was investigated in *B. subtilis* 1.2172 obtained from China General Microbial Culture Preservation Management Center (China). It was inoculated in liquid Tryptone soya broth (TSB) medium containing increasing Sb concentrations (0, 300, 600, 900, and 1200 mg L^{-1}) and incubated at 30 °C at 200 rpm for 7 d to determine Sb tolerance potential [51]. The optical density was measured every day, and an optical density of 0.5 was considered indicative of the strain exhibiting tolerance to Sb [52], *B. subtilis* 1.2172 grew

exponentially and showed tolerance against all the tested Sb concentrations.

2.2. Biofilm, indole-3-acetic acid, ACC deaminase, and siderophore production in Bacillus subtilis 1.2172 at varying Sb concentrations

To further test *Bacillus subtilis* 1.2172 tolerance to Sb, this strain was grown in TSB medium added with increasing Sb concentrations (0, 300, 600, 900, and 1200 mg L^{-1}) for 72 h at 30 °C and tested for the following features: (i) biofilm forming ability of *B. subtilis* 1.2172 was determined with the method of O'Toole and Kolter [53] (ii) indole-3-acetic acid (IAA) concentration was determined with the method of Loper and Schroth [54] (iii) ACC deaminase and exopolysaccharides were determined according to Ali et al. [55]; siderophore production was assessed with the method of Schwyn and Neilands [56].

2.3. Soil preparation and experimental treatments

The soil used in the study was collected from a paddy field of the experimental station of Jiangxi Agricultural University (28° 27′ N, 115° 36′ E; 25 m above sea level) in Nanchang, China. The experimental field had served for rice-rapeseed rotation for several years. The experimental site has a sub-tropical monsoon humid climate with sufficient sunshine (6330 MJ m^{-2}), 26.7 °C average temperature, and 14.1 mm average daily rainfall during rice growing season. The collected paddy soil was subjected to air drying, sieving (2 mm), thoroughly mixed and submitted to the determination of different soil properties. The soil had a silty-loamy texture (sand 24 %, silt 56 %, and clay 20 %) with pH 5.42, CEC 7.20 cmol kg^{-1}, organic carbon 11. 62 g kg^{-1}, total nitrogen 1.56 g kg^{-1}, C:N 7.4, available phosphorus 26 mg kg^{-1} and exchangeable potassium 108 mg kg^{-1}.

The study comprised six treatments: control (Sb free soil), Sb contaminated soil (1200 mg Sb kg^{-1}), Sb contaminated soil (1200 mg Sb kg^{-1}) + BC (1 %), Sb contaminated soil (1200 mg Sb kg^{-1}) + BC (2.5 %), Sb contaminated soil (1200 mg Sb kg^{-1}) + BC (1 %) + BI and Sb contaminated soil (1200 mg Sb kg^{-1}) + BC (2.5 %) + BI. The concentration of Sb was used at the rate of 1200 mg kg^{-1} which in accordance with Sb concentration in polluted soils [1565 mg kg^{-1}] in China [4]. The experiment was set up in a completely randomized design with three replicates. The soil Sb concentration was validated by inductively coupled plasma-mass spectrometry following digestion.

The pots were filled with 8 kg of soil on a dry weight basis. The Sb concentration was obtained by adding $C_8H_4K_2O_{12}Sb_2$ (analytical grade) to the soil, followed by water supply to attain 70 % field capacity. The soil was subsequently left ageing prior to use for the experiment. Four weeks later, each pot was emptied and the soil was placed on a plastic sheet, BC with/without BI was mixed with soil at 1 % and 2.5 %, and pots were filled again. On April 28, 2023, five rice seedlings were transplanted into each pot, pots were regularly visited, and irrigation was applied whenever needed to maintain 70 % field capacity. The rest of the management practices were carried out with due care to get a good stand establishment and subsequent growth. Rice plants were harvested at physiological maturity on July 28, 2023. The samples for determining different traits were collected and handled with great care to obtain reliable results. The standard procedures with fully sterilized materials and instruments were used to determine different traits. Additionally, analytical grade chemicals provided by Sigma-Aldrich were used to measure different parameters.*2.4. Biochar production and bacterial inoculation.*

Maize straw was submitted to pyrolysis at 600 °C for 8 h under limited oxygen supply and heating rate of 5 °C min^{-1} to produce biochar. Then BC was sieved (2 mm), and a series of properties was determined. The prepared BC had pH 9.90, carbon content 640 g kg^{-1}, CEC 10.97 cmol kg^{-1}, N content 4.52 g kg^{-1} and C:N 142. For the preparation of bacterial inoculated BC, the strain *Bacillus subtilis* 1.2172 was cultivated in nutrient broth (NB) (0.3 % of beef extract and 0.5 % of peptone) with an inoculum size of 5 % at 30 °C for 24 h and then centrifuged at 200 rpm until OD-600 value reached 1. BC and bacterial culture were blended at the rate of 1:3 (*w/v*) and incubated for 8 h prior to use for the experiment. Samples of BC and bacterial inoculated BC were submitted to scanning electron microscope (SEM) analysis in order to detect structural differences between the two products.

2.4. Photosynthetic pigments and leaf water contents

To determine plant physiological and biochemical traits, plant samples were collected at flag leaf stage (12 June 2023). Leaf fresh samples were taken, and concentrations of chlorophyll *a*nd carotenoids were measured using the methods of Arnon [57]. 0.5 g leaf fresh samples were homogenized using 80 % acetone, then the extract was centrifuged, and absorbance of the supernatant was taken at 663, 645, and 470 nm for the determination of chlorophyll *a*, chlorophyll *b,* and carotenoids concentration, respectively. In the case of anthocyanin, 0.5 g of fresh leaves were taken and homogenized with potassium phosphate buffer (PPB: 5 mL). Then, an extract was obtained, centrifuged for 15 min, and absorbance was taken at 535 nm [58]. In the case of relative water content (RWC), fresh leaves were taken and weighed (FW), then they were sub-merged in water for 24 h in the dark. Leaves were then taken, excessive water was removed, and turgid weight (TW) was determined. Lastly, the samples were dried in an oven (70 °C), and the dry weight (DW) was assessed. RWC was determined with the following equation: RWC = (FW-DW)/(TW-DW) × 100.

2.5. Oxidative stress markers, osmo-regulating compounds, and antioxidant activities

Leaf fresh leaf samples (0.5 g) were taken and then ground with trichloroacetic acid (TCA: 5 L) and centrifuged. Then, the extract was obtained, and 1 mL was placed in 100 μL of PPB in water for 30 min. After that, absorbance was read at 390 nm to determine H_2O_2 concentration [59]. The concentration of malondialdehyde (MDA) was measured with the procedure of Rao and Sresty [60]. 0.5 g fresh plant sample was ground in 5 mL of TCA and centrifuged at 12000 rpm for 15 min. A 1 mL extract mixed with 1 mL TCA was placed in a water bath at 100 °C for 30 min. Then, it was rapidly cooled, and absorbance was read at 532 to determine MDA concentration. To assess electrolyte leakage (EL), 0.5 g fresh sample was divided into pieces and placed in water, and the first EC was taken (EC1). Afterward, these samples were placed in a water bath for 24 h at 90 °C, and second EC was recorded (EC2). Finally, EL was measured with the equation: EL = $EL_1/EC_2 \times$ 100.

In the case of free amino acids (FAA), leaf fresh samples (0.5 g) were ground with 5 mL of PPS and centrifuged for 15 min at 15000 rpm, and the extract was obtained. One mL of extract was taken and 1 mL of ninhydrin and pyridine were added and placed at 9 0 °C for 30 min and then allowed to cool. Afterwards, absorbance was read at 570 nm to determine FAA concentration [61]. For determining total soluble proteins (TSP); 0.5 g fresh samples were ground with 5 mL of PPB and centrifuged for 15 min at 15000 rpm, and extract was obtained. Thereafter, 1 mL extract was added with 3 mL of Bradford reagent and placed at room temperature for 15 min, and absorbance was read at 595 nm [62]. To determine proline concentration, 0.5 g leaf samples were taken, ground with 10 mL sulfosalicylic acid (3 %), and centrifuged at 10000 rpm for 15 min. Then, the supernatant was mixed with acid-ninhydrin and placed at 90 °C for 30 min. The absorbance was read at 520 nm [63].

The catalase (CAT) activity was measured with the method of Chance and Maehly [64]. Fresh leaf samples (0.5 g) were homogenized in 5 mL of PPB and then centrifuged for 10 min at 12000 rpm, and absorbance was read at 240 nm [64]. For the determination of peroxidase (POD) activity, the procedure of Guan et al. [65] was used. Briefly, 0.5 g leaf samples were ground with 5 mL of PPB and centrifuged for 10 min at 12000 rpm, and absorbance was read at 470 nm. The activity of

ascorbate peroxidase (APX) was measured with the procedure of Nakano and Asada [66]. The plant extract was obtained, and 100 μL extract was mixed with 100 μL ascorbate and 100 μL H_2O_2, thereafter absorbance was read at 290 nm. In the case of super-oxide dismutase (SOD), 0.5 g fresh leaf samples were taken, and 3 mL reaction solution was prepared by adding 50 μL riboflavin, 50 μL nitro blue tetrazolium, 100 μL L-methionine, 50 μL enzyme extract, 100 μL triton-X. After mixing, the reaction mixture was added to a test tube, and the absorbance was read at 560 nm [66].

2.6. Plant growth and yield traits

Five plants at maturity were carefully up-rooted, and roots were separated from above-ground parts. Roots were weighed to determine fresh weight, then they were oven (70 °C) dried to determine dry weight. The height of five plants in each pot was measured and averaged to determine plant height; similarly, fertile tillers from every plant of each pot were counted, and the average was taken. Ten panicles were randomly selected from each pot to determine their length and the number of kernels per panicle. The complete pots were harvested and weighed to determine biological yield, and they were threshed to measure grain yield. Moreover, a sub-sample of rice kernels was taken to assess the numbers of total, sterile and abortive kernels.

2.7. Antimony concentration in plant parts

On plant samples at harvest, and roots, stems, leaves and rice grains were separated and dried. After that, 100 mg of each plant part was placed in digestion tubes with 2 mL of HNO_3. Then, the digestion tubes were digested for 2 h at 150 °C until all the material was completely digested. The tubes were subsequently left to cool at room temperature and placed in a water bath at 90 °C until the solution became clear. Thereafter, the solution was diluted using 1 % HNO_3, and the volume was made to 50 mL filtered through 0.45-μm polyether sulfone membrane and analyzed with inductively coupled plasma source mass spectrometer (iCAP Q/Qnova series, ICAPRQ00509, Thermo Fisher Scientific) to determine the concentration of Sb. This instrument is operated at an RF power of 1550 W with nebulizer gas flowing at the rate of 15 L/min. The accuracy of the method was confirmed by using reference material of Sb provided by Sigma-Aldrich. Additionally, all the samples were analyzed in triplicate to get reliable results.

2.8. Antimony bio-accessibility

In vitro Sb bio-accessibility in the gastric phase was measured with the SBET method [67]. 1 g soil samples were taken and extracted with 0.4 mol/L glycine juice. Thereafter, this mixture was placed for 1 h at 30 rpm in a thermostat (37 °C) at a pH of 1.5 which was maintained by means of HCl addition. Then the mixture was filtered with 0.45 μm filter paper [68], and Sb concentration was measured with atomic absorbance spectrophotometry (Hitachi, Model-7JO8024, Tokyo, Japan). The bio-accessibility of Sb (BioSb) was determined with the following equation:

$$BioSb = \frac{SbBio}{TSB} \times 100$$

Herein, TSB indicates total Sb, S SbBio and BioSb indicate the respective bio-accessible concentration of Sb and bio-accessibility of Sb in the gastric environment.

2.9. Soil chemical, biological, and enzymatic properties

The pH of the experimental soil after harvesting the rice plants was measured with a pH meter (1:5 soil: distilled water). To determine soil microbial biomass carbon (MBC), 10 g of moist soil was taken in two sets. From these two sets, one set was fumigated with chloroform, and the other set was not fumigated with chlorophyll for 24 h. Thereafter, both sets of soils were extracted with 50 ml of K_2SO_4 (0.5 m) and filtered with filtered paper, and the concentration of carbon was measured with a carbon analyser. On the other hand, soil N, and available P, K were determined with the Kjeldahl method, sodium bicarbonate extraction (spectrophotometry), and ammonium acetate extraction flame photometry methods. Urease and catalase activities were assessed and expressed as mg NH_4-N g^{-1} day^{-1} and μmol H_2O_2 g^{-1} day^{-1}, respectively. Soil samples were taken, and ground and total Sb (TSb) concentration was measured using the strong acid pseudo total digestion method. Briefly, 500 mg soil was added with HCl (6 mL) HNO_3 (2 mL) in a Teflon bomb and placed in a water bath (>95 °C). Thereafter, this solution was allowed to cool, the volume was 50 mL, and the TSb was determined with atomic absorbance spectrophotometry.

2.10. Soil bacterial community diversity

After rice was harvested at physiological maturity, the 0–20 cm topsoil was randomly taken from each pot by the "five-point sampling method". After mixing, it was immediately frozen in liquid nitrogen and stored at −80 °C. The collected samples represent the six experimental treatments described in Table S1. The sample was commissioned by Meiji Biomedical Technology Co. Ltd. (China) for high-throughput sequencing. The main steps are as follows: firstly, the microbial DNA in the soil sample was extracted using the DNeasy Power Soil Pro Kit from QIAGEN (USA). Then, the DNA concentration and purity were determined by an ultra-micro spectrophotometer (Nanodrop ND-1000), and DNA integrity was detected by 1 % agarose gel electrophoresis. Primer 338F: ACTCCTACGGGGAGGCAGCAG and primer 806R: GGACTACHVGGGTWTCTAAT were used to amplify the 16S rRNA of microorganisms. The products were detected by 2 % agarose gel electrophoresis. The PCR products were identified, purified, and quantified, and the Miseq library was constructed. Finally, the Illumina company's Miseq PE300 platform was used for sequencing.

The raw sequence data were quality-checked through FastQC [69]. The sequences were pre-processed, quality-filtered, trimmed, de-noised, merged, and modelled using DADA2, including removing chimeric sequences, all performed within QIIME2 [70]. The next step involved clustering amplicon sequence variants (ASV) into operational taxonomic units (OTUs) with a 97 % cut-off using VSEARCH [71], which was also performed within QIIME2. Representative OTUs were taxonomically assigned using a Naïve–Bayes classifier trained SILVA SSU 138.1 [72]. All sequences have been submitted to the NCBI under the study accession number PRJNA1120056.

2.11. Statistical analysis

A completely randomized factorial design with three replications was adopted as experimental scheme. Normal distribution (Kolmogorov-Smirnov test) and homogeneity of variances (Bartlett's test) were assessed in all soil and plant traits. The data were submitted to one-way ANOVA by means of the CoStat 6.451 software (CoHort Software, Monterey, CA, USA). Tukey's test at $P < 0.05$ was used to separate means of significant traits.

Additionally, data were analyzed by the orthogonal contrast method (or single degree of freedom procedure) [73] to test the effects of selected treatment combinations to answer a priori (i.e., planned) questions. Specifically, five contrasts were set up (Table S2): linear response to BC (0, 1 %, 2.5 % under Sb stress); quadratic response to BC; BI vs. no BI; BI×BC interaction; and stress abatement, i.e., unstressed control vs. maximum stress relief, this latter consisting of BC (2.5 %) + BI. The BI×BC interaction, which only in a few traits was shown to be significant, was dismissed in subsequent tables.

Bacterial beta diversity was investigated via non-metric multidimensional scaling (NMDS) and permutational multivariate ANOVA (PERMANOVA) using the vegan R package [74]. The Maaslin2 R package was used to analyse differences in taxonomic composition

among the different samples [75].

3. Results

3.1. Bacillus subtilis 1.2172 tolerance to Sb

Bacillus subtilis 1.2172 was able to grow up to the top Sb concentration (1200 mg L^{-1}) (Fig. S1). The time trend in all treatments showed an increase in OD ($P \leq 0.05$) up to peak values on the 4th day and a subsequent decline (Fig. S1). At peak time, the maximum OD level was observed in the control, while the minimum OD was observed at 1200 mg L^{-1} Sb concentration (Fig. S1). The highest Sb concentration (1200 mg L^{-1}) at which *B. subtilis* was still able to grow was subsequently used to test its ability to detoxify the soil where plants were grown.

Antimony concentration significantly ($P \leq 0.05$) influenced the ability of *B. subtilis* 1.2172 to form biofilm (Fig. 1). The control exhibited minimal biofilm formation, while the highest biofilm formation occurred in the presence of 300 mg L^{-1} of Sb. A linear decrease in biofilm formation was observed from 300 to 1200 mg L^{-1} of Sb, resulting in a 70 % decrease at 1200 mg L^{-1} with respect to 300 mg L^{-1}. Indole-3-acetic acid and siderophore concentrations significantly increased with increasing Sb toxicity, and the maximum concentration of both IAA and siderophore was seen at 1200 mg L^{-1} Sb (Fig. 1). Likewise, the concentration of ACC and *exo*-polysaccharide also showed an increasing trend with increasing Sb concentration (Fig. 1). The maximum ACC and exo-polysaccharide concentrations were recorded at 1200 mg L^{-1} Sb, while the lowest were observed in the control (Fig. 1).

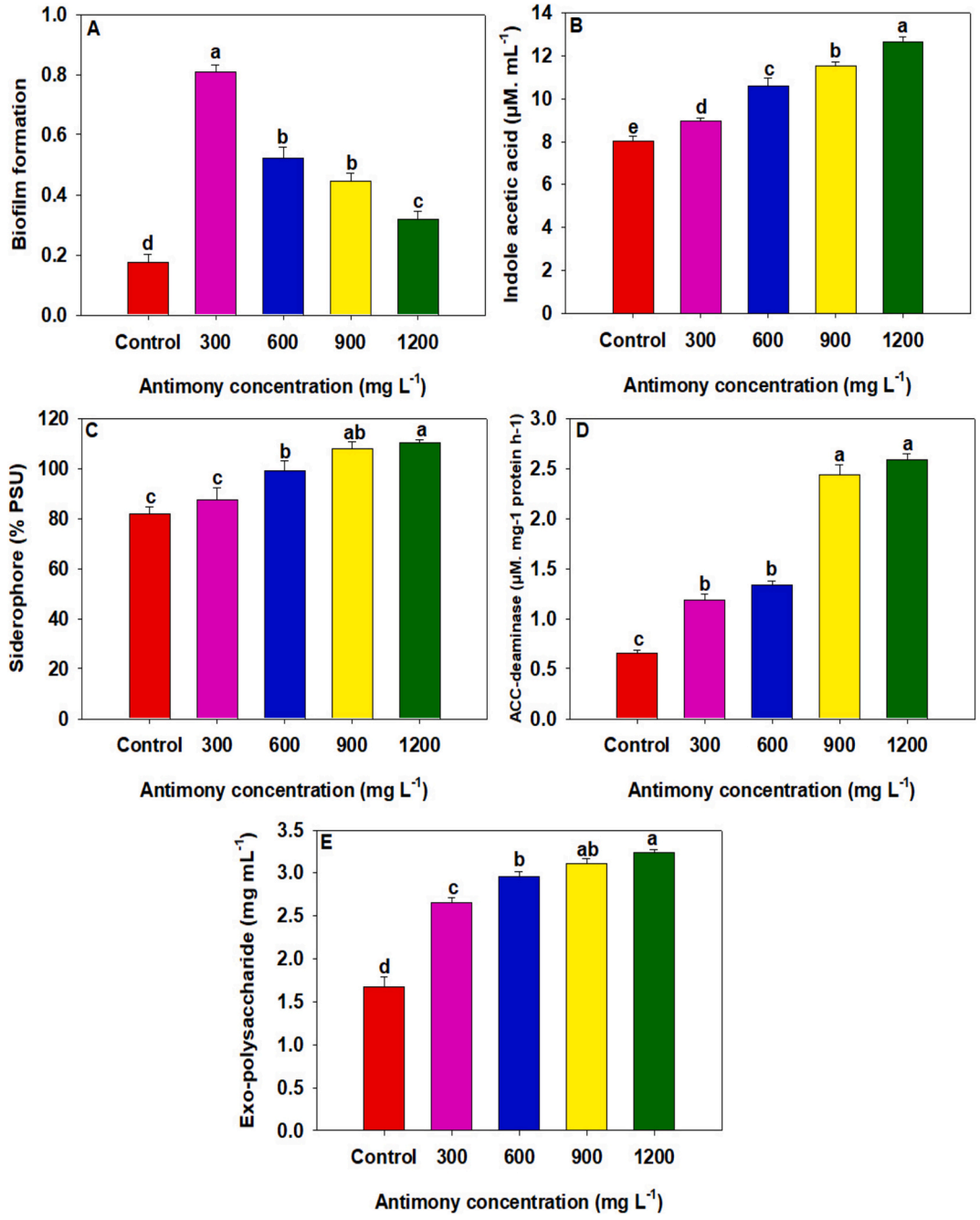

Fig. 1. Effect of different antimony concentrations on biofilm formation (A), indole acetic acid (B), siderophore (C), ACC-deaminase (D) and *exo*-polysaccharide (E) production of *B. subtilis* 1.2172. The data indicate the means ($n = 3$), and different letters indicate statistical differences at $P \leq 0.05$).

3.2. Scanning electron microscope analysis

The SEM analysis of biochar and bacterial-inoculated biochar showed significant differences between the two samples (Fig. S2). The biochar alone showed a rough surface with a porous structure (Fig. S2a). The bacterial-inoculated biochar revealed a high presence of adhered bacterial cells on the biochar: most bacterial cells were well scattered on the biochar surface, with some of them embedding in the pore structures of the biochar as well (Fig. S2b).

3.3. Plant physiological and biochemical activities at flag leaf stage

3.3.1. Leaf water status and photosynthetic pigments

Leaf water status ($P \leq 0.01$) and photosynthetic pigments ($P \leq 0.05$) exhibited sizeable differences in treatment comparisons (Table 1). The RWC showed a linear increase upon BC supply (+ 29 % with 2.5 % BC), a significant BI effect (+13 %), and the combined ability of 2.5 % BC + BI to recover Sb stress, as the insignificant difference between Unstressed and Maximum stress relief demonstrates. The EL showed a significant quadratic decrease with BC supply (−53 % with 2.5 % BC), indicating that the strong decrease with the lower BC dose (1 %) hardly improved with the higher dose (2.5 %). The EL was also significantly ($P \leq 0.05$) decreased with BI addition to BC (−19 %). Lastly, the maximum stress containment with 2.5 % BC + BI was shown to be statistically at par with the unstressed control, indicating that the efficiency of cell membranes was substantially restored.

The four photosynthetic pigments (chlorophyll *a* and *b*, carotenoids, and anthocyanin) improved upon BC supply (average, +30 % with 2.5 % BC) according to a linear (chlorophyll *b* and anthocyanin) or quadratic response (chlorophyll *a* and carotenoids). They all significantly increased with BI (average, +11 %) and showed, once more, a remarkable stress recovery with the maximum stress relief compared to the unstressed control (Table 1). In fact, only in chlorophyll *b* a statistical difference was evidenced between these two treatments.

3.3.2. Osmo-regulating compounds, antioxidant enzymes, and oxidative stress markers

These different compound categories also staged significant variations in treatment comparisons (Table 1). The three osmo-regulating compounds, TSP ($P \leq 0.05$), FAA ($P \leq 0.05$), and proline ($P \leq 0.05$), increased upon BC supply (average, +40 % with 2.5 % BC): TSP and proline showed a linear response trend, whereas FAA outlined a quadratic trend indicating a near plateau effect at the higher (2.5 %) BC level. All of them increased to a similar extent with BI addition to BC (average, +17 %). Lastly, with the maximum stress relief treatment (2.5 % BC + BI), none of the three osmo-regulating compounds attained the levels of the unstressed control, indicating a partial recovery (Table 1).

The four antioxidant enzymes, CAT, POD, SOD, and APX, linearly ($P \leq 0.05$) increased at increasing BC levels (average, +57 % with 2.5 % BC). They also increased with BI addition to BC (average, +26 %). Lastly, with the maximum stress relief, the four antioxidant enzymes attained the highest levels in contrast to the low levels of the control (Table 1).

The two oxidative stress markers MDA and H_2O_2 decreased ($P \leq 0.05$) with a quadratic response trend at increasing BC levels (average, −41 % with 2.5 % BC). They also decreased upon BI addition to BC (average, −12 %). Lastly, with the maximum stress relief treatment MDA attained the low level of the unstressed control, whereas H_2O_2 remained statistically differentiated (Table 2).

3.3.3. Morphological and yield traits at harvest

Morphological and yield traits ($P \leq 0.05$) were also significantly influenced by Sb stress, BC, and BI (Table 2). Plant height increased according to a quadratic trend in response to BC doses (+31 % with 2.5 BCE). Plant height also increased with BI addition to BC (+14 %). However, the maximum stress relief did not attain the plant height of the unstressed control, indicating that the stress effect could not be fully recovered.

The three yield components, i.e., number of fertile tillers per plant, number of kernels per panicle, and thousand kernel weight, behaved inconsistently. Tillers per plant did not significantly ($P \leq 0.05$) respond to BC but only to BI addition (+11 %). Conversely, the other two components responded to BC according to a linear (kernels per panicle) and quadratic trend (thousand kernel weight) (average, +35 % with 2.5 % BC). Both traits benefitted from BI addition to BC (average, +16 %), and both of them exhibited only a partial recovery with the maximum stress relief vs the control.

The grain yield ($P \leq 0.05$), based on the components mentioned above, exhibited a linear response to BC (+69 % with 2.5 % BC) and an increase upon BI addition (+28 %). As in the case of previous traits, the maximum stress relief did not attain the grain yield of the unstressed control. The harvest index, i.e., the dry weight ratio between total aboveground biomass (not shown) and grain yield, increased linearly in response to BC (+19 % with 2.5 % BC), also increased with BI addition (+7 %), and with the maximum stress relief attained at the same statistical level as the control. Finally, the root to shoot dry weight ratio

Table 1
Orthogonal contrasts in leaf water status, photosynthetic pigments, antioxidant activities and osmo-regulating compounds in rice at flag leaf stage.

Treatment comparisons	RWC (%)	EL (%)	Chl. *a* (mg g^{-1} FW)	Chl. *b* (mg g^{-1} FW)	Cart. (mg g^{-1} FW)	Anth. (mg g^{-1} FW)	CAT (U mg^{-1} prot.)	POD (U mg^{-1} prot.)	SOD (U mg^{-1} prot.)	APX (U mg^{-1} prot.)	TSP (mg g^{-1} FW)	FAA (mg g^{-1} FW)	Proline (mg g^{-1} FW)
Bio-char (BC)													
0	56.0	43.7	0.40	0.27	3.12	7.0	2.76	0.14	2.08	6.57	7.2	6.2	0.72
1 %	63.3	24.7	0.50	0.31	3.83	7.8	3.24	0.17	2.55	7.15	8.2	8.1	0.79
2.5 %	68.0	23.7	0.50	0.33	3.95	8.1	3.92	0.22	2.87	7.79	8.8	9.1	0.84
Linear response	**	**	**	**	**	**	**	**	**	**	**	**	*
Quadratic response	ns	**	**	ns	**	ns	ns	ns	ns	ns	ns	*	ns
Bio-inoculant (BI)													
No	65.7	24.2	0.50	0.32	3.89	7.9	3.58	0.20	2.71	7.47	8.5	8.6	0.82
Yes	74.3	19.5	0.55	0.35	4.28	9.1	4.45	0.27	3.43	8.53	10.1	10.2	0.92
Contrast	**	**	**	**	**	**	**	**	**	**	**	**	**
Stress abatement													
Unstressed	81.0	12.3	0.60	0.42	4.54	11.3	2.42	0.10	1.59	5.71	13.7	14.7	0.55
Max. stress relief	77.0	17.7	0.56	0.37	4.43	9.3	4.63	0.30	3.64	8.75	10.6	10.5	0.95
Contrast	ns	ns	ns	*	ns	**	**	**	**	**	**	**	**

Maximum stress relief, 2.5 % BC + BI; RWC, relative water content; EL, electrolyte leakage; MSI, membrane stability index; Chl. *a* and Chl. *b*, chlorophyll *a* and *b*, respectively; FW, fresh weight; Cart: carotenoids, Anth: anthocyanin. CAT, catalase; POD, peroxidase; SOD, super-oxide dismutase; APX, ascorbate peroxidase. n.s., * and ** indicate non-significant and significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

Table 2
Orthogonal contrasts in oxidative stress markers at flag leaf stage, and morphological and yield traits in rice at physiological maturity.

Treatment	MDA (μmol g^{-1} FW)	H_2O_2 (μmol g^{-1} FW)	Plant height (cm)	Tillers/ plant	Kernels/ panicle	TKW (g)	GY (g $plant^{-1}$)	HI	R:S
Bio-char (BC)									
0	8.02	4.29	61.0	6.7	57.7	19.9	15.5	0.40	0.15
1 %	4.86	3.00	69.7	7.3	66.0	24.0	19.4	0.42	0.14
2.5 %	4.76	2.83	74.7	7.7	71.3	25.1	23.7	0.47	0.14
Linear response	**	**	**	ns	**	**	**	**	ns
Quadratic response	**	**	*	ns	ns	**	ns	ns	ns
Bio-inoculant (BI)									
No	4.81	2.92	72.2	7.5	68.7	24.6	21.6	0.44	0.14
Yes	4.43	2.45	82.5	8.3	79.8	28.4	27.6	0.47	0.14
Contrast	**	**	**	*	**	**	**	*	ns
Stress abatement									
Unstressed	4.10	1.47	92.0	9.0	85.7	30.1	33.0	0.50	0.15
Max. stress relief	4.31	2.39	85.0	8.7	83.7	28.9	28.7	0.48	0.14
Contrast	ns	**	**	ns	**	*	**	ns	ns

Maximum stress relief, 2.5 % BC + BI; MDA, malondialdehyde; H_2O_2, hydrogen peroxide; TKW, thousand kernel weight; GY, grain yield; HI, harvest index; R:S, root to shoot ratio; FW, fresh weight; n.s., * and ** indicate non-significant and significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

was not influenced by any treatment combination (Table 2).

3.3.4. Antimony uptake and partitioning to plant organs

Antimony concentration in plant organs exhibited ($P \leq 0.05$) a different picture, in general, with respect to Sb content in plant organs and the whole plant (Table 3). In all four organs (roots, stems, leaves, and grain) and the total plant composition, the Sb stress treatment evidenced the highest Sb concentrations. The four combinations of BC and BI determined a reduction in Sb concentration, which was proportional to BC and BI supply. As a result, the 2.5 % BC + BI treatment provided the strongest abatement in Sb concentrations (−43 % in the total plant composition). In exchange for this, Sb content was statistically ($P \leq 0.05$) undifferentiated in all organs except leaves, owing to the fact that progressive mitigation in the four BC and BI treatments was associated with a concurrent increase in the biomass of the four organs and the whole plant (this last effect reported in Table 3). As a result, the total Sb content, i.e., the removal from the soil, was significantly reduced only in the two treatments involving the higher BC dose (i.e., 2.5 % BC with/without BI) (average, −12 %). The Sb partitioning among plant organs slightly varied among treatments: of the total Sb content, an average of 45 % was retained in roots, 48 % in stems, 4 % in leaves, and only 3 % in grains.

Based on Sb supply and plant retrieval, the Sb bio-accumulation factor indicating Sb whole plant concentration with respect to soil concentration, significantly decreased in response to BC and BI from 1.51 mg kg^{-1} of applied Sb under Sb stress, to 0.86 mg kg^{-1} with 2.5 % BC + BI (Table 3).

3.3.5. Antimony bio-accessibility

Total and bio-accessible Sb concentrations in the gastric phase were strongly reduced by BC doses and BI addition (Fig. 2). However, BC and BI exerted a stronger influence on the bio-accessible fraction than on the total content. This is clearly shown by the decreasing bio-accessibility, according to which the bio-accessible Sb decreased from 16.4 % of the total element under Sb stress to 10.9 % with 2.5 % BC + BI (Fig. 2).

3.3.6. Soil traits

Soil chemical, biological, and enzymatic parameters at harvest were variously significantly ($P \leq 0.05$) influenced by experimental treatments (Table 4). The acidic pH of this soil (5.38 in the control) was not influenced by Sb input. BC supply to mitigate Sb stress remarkably increased soil pH with a linear trend. BI addition significantly raised soil pH, too. As a consequence, the maximum stress relief involving 2.5 % BC + BI improved soil pH by almost 0.6 towards neutrality, which is an unexpected yet favorable side effect, besides the mitigation of Sb stress.

The two plant available nutrients, P and K, behaved similarly. They increased with a linear trend at increasing BC doses and positively responded to BI addition. However, even with the maximum stress relief, the two nutrients did not attain the same levels as the control. In the case of K, which was actually deficient in this acidic soil, the gap with the unstressed control was less than 15 %.

Total soil N and soil organic C increased with a linear ($P \leq 0.05$) trend at increasing BC doses and were also positively influenced by BI addition. The differences between maximum stress relief and unstressed control were insignificant, indicating for both traits a complete recovery from Sb stress. The C:N ratio was rather high for an agricultural soil, owing to the fact that the soil was quite rich in SOC (19.1 g kg^{-1} in the control). BC supply further augmented the C:N ratio following a linear trend; BI addition significantly reduced it. Lastly, the combined BC + BI in the maximum stress relief evidenced a C:N slightly, yet insignificantly higher than the unstressed control.

Soil microbial biomass carbon increased upon BC supply following a quadratic trend; MBC significantly increased following BI addition. Lastly, MBC in the maximum stress relief was shown to be quite higher

Table 3
Antimony (Sb) concentrations (mg kg^{-1} DW) and contents (μg $plant^{-1}$) in rice organs and the whole plant at physiological maturity, and bio-accumulation factor.

Organ	Root	Stem	Leaf	Grain	Total	Plant DW	Root	Stem	Leaf	Grain	Total	BAF
Treatment	(mg kg^{-1} DW)					(g $plant^{-1}$)	(μg $plant^{-1}$)					(mg kg^{-1})
Sb stress	**64.2^{a}**	**18.5^{a}**	**12.8^{a}**	**1.36^{a}**	**18.2^{a}**	**44.9^{d}**	**366.9**	**400.8**	**25.4^{b}**	**21.1**	**814.3^{a}**	**1.51^{a}**
Stress + 1 % BC	**50.5^{b}**	**15.8^{b}**	**11.0^{b}**	**0.98^{b}**	**14.4^{b}**	**53.1^{c}**	**331.4**	**381.7**	**33.9^{a}**	**19.1**	**766.1^{ab}**	**1.20^{b}**
Stress + 2.5 % BC	**46.5^{bc}**	**14.4^{bc}**	**9.6^{c}**	**0.92^{bc}**	**12.5^{c}**	**57.9^{b}**	**330.1**	**341.2**	**32.3^{a}**	**21.7**	**725.4^{b}**	**1.04^{c}**
Stress + 1 % BC + BI	**42.6^{c}**	**12.9^{cd}**	**8.9^{c}**	**0.85^{c}**	**11.4^{cd}**	**64.7^{a}**	**342.8**	**341.6**	**32.8^{a}**	**22.5**	**739.7^{ab}**	**0.95^{cd}**
Stress + 2.5 % BC + BI	**37.8^{d}**	**12.1^{d}**	**7.7^{d}**	**0.71^{d}**	**10.3^{d}**	**68.7^{a}**	**323.8**	**329.5**	**32.0^{a}**	**20.3**	**705.6^{b}**	**0.86^{d}**
P	**< 0.01****	**< 0.01****	**< 0.01****	**< 0.01****	**< 0.01****	**< 0.01****	**0.37 n. s.**	**0.16 n. s.**	**< 0.01****	**0.11 n. s.**	**0.02***	**< 0.01****

BC, bio-char; BI, bio-inoculant; DW, dry weight; BAF, bio-accumulation factor; n.s., * and ** indicate non-significant and significant at $P \leq 0.05$ and $P \leq 0.01$, respectively. Different letters indicate statistical differences (Tukey test at $P \leq 0.05$).

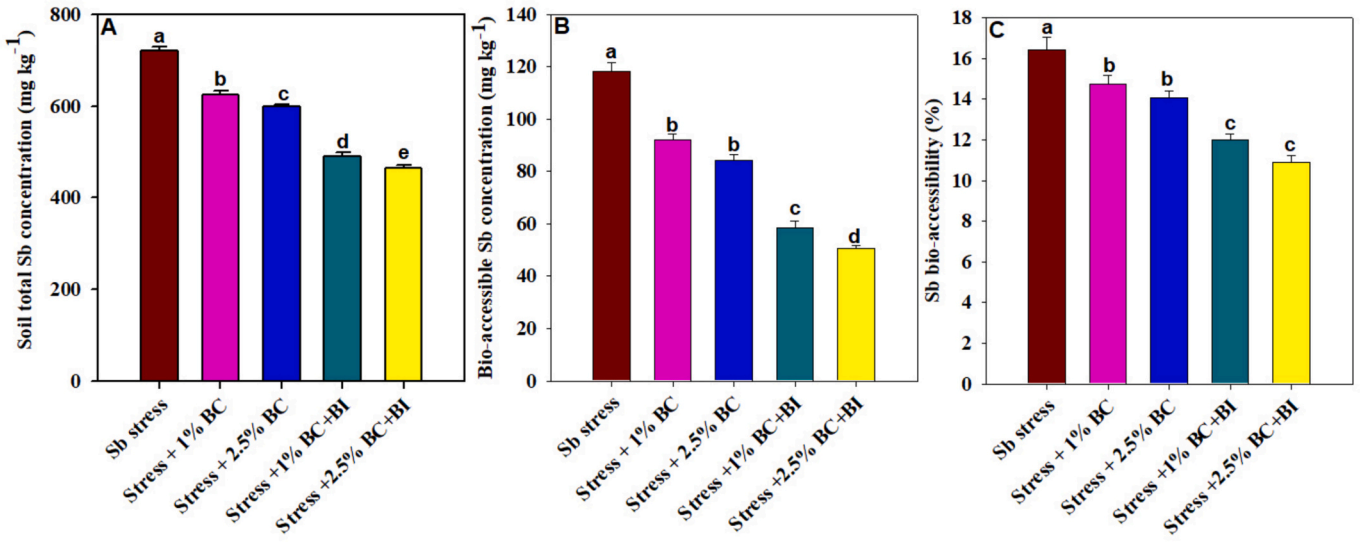

Fig. 2. Effect of BC and BI on soil total Sb concentration (A), bio-accessible Sb concentration (B) and bio-accessibility of Sb (C). The data indicate the means ($n = 3$), and different letters indicate statistical differences (Tukey test at $P \leq 0.05$).

Table 4
Orthogonal contrasts in soil traits at rice harvest.

Treatment	pH	Available P (g kg^{-1})	Exch. K (g kg^{-1})	TN (g kg^{-1})	SOC (g kg^{-1})	C:N	MBC (mg kg^{-1})	MBC % SOC	Urease (mg NH_4-N g^{-1} d^{-1})	Catalase µmol H_2O_2 g^{-1} d^{-1})
Bio-char (BC)										
0	5.35	14.7	63	0.75	14.0	19.0	355	2.5	0.30	9.6
1 %	5.53	16.6	73	0.89	17.5	19.7	413	2.4	0.37	11.1
2.5 %	5.69	17.9	79	0.98	19.7	20.1	426	2.2	0.40	13.1
Linear response	**	**	**	**	**	**	**	**	**	**
Quadratic response	ns	ns	ns	ns	ns	ns	**	ns	ns	ns
Bio-inoculant (BI)										
No	5.61	17.3	76	0.94	18.6	19.9	420	2.3	0.39	12.1
Yes	5.91	20.2	84	1.20	21.9	18.2	474	2.2	0.49	15.6
Contrast	**	**	**	**	**	ns	**	ns	**	**
Stress abatement										
Unstressed	5.38	26.9	97	1.25	19.1	15.3	388	2.0	0.64	19.2
Max. stress relief	5.95	20.7	85	1.26	22.6	18.0	487	2.2	0.52	16.3
Contrast	**	**	**	ns	ns	ns	**	ns	**	**

Maximum stress relief, 2.5 % BC + BI; TN, total nitrogen; SOC, soil organic carbon; C:N, SOC/TN; MBC, microbial biomass carbon; n.s., * and ** indicate non-significant and significant at $P \leq 0.05$ and $P \leq 0.01$, respectively.

than the unstressed control. Based on MBC and SOC response to experimental treatments, the proportion of MBC to SOC linearly decreased at increasing BC doses, whereas it was not significantly influenced by BI addition. Nor was it statistically ($P \leq 0.05$) different between maximum stress relief and control. The two soil enzymes, urease and catalase, behaved in a similar way. They increased with a linear trend at increasing BC doses and positively responded to BI addition. However, even with the maximum stress relief both enzymes remained between 15 % and 20 % below the levels evidenced by the unstressed control (Table 4).

3.4. Soil bacterial community richness, composition and diversity

3.4.1. Bacterial richness and diversity indices

The circumstance that each sample curve approached a plateau suggests that the sequencing depth was sufficient to describe the variability within the microbial communities of the 18 samples obtained from the 6 treatments and 3 replicates. The DADA2 pipeline identified 38 different phyla, 372 families, and 494 genera among all samples. The relative abundance of the ten most abundant taxa at the phylum level is shown in Fig. 3.

3.4.2. Bacterial community composition

Analyses of 16S rRNA data showed all the treatments are able to induce a significant ($P \leq 0.05$) smodulation of the soil microbiota composition at the phylum level. The top 10 phyla with higher richness are Acidobacteriota, Bacteroidota, Chloroflexi, Firmicutes, Gemmatimonadota, Myxococcota, Patescibacteria, Planctomycetota, Proteobacteria, Verrucomicrobiota (Fig. 4). Proteobacteria and Firmicutes increased in treatment with BC and with BC + BI.

The most abundant taxa that were found in CTRL are *Gemmatimonas* (7.8 %), *Chitinophagaceae* (4.6 %), SC-1-84 (3.9 %), *Candidatus Solibacter* (3.7 %) and RBG-13-54-9 (3.7 %). All of these taxa are commonly recognized as PGPR (Plant Growth Promoting Rhizobacteria) in soil. Sb stress +1 % BC positively modulated *Sphingomonas* (4.2 %), *Chitinophagaceae* (3.2 %), *Flavisolibacter* (2.4 %), *Candidatus Solibacter* (1.9 %) and *Tellurimicrobium* (1.7 %). Notably, *Sphingomonas, Flavisolibacter* and *Tellurimicrobium* are recognized for their ability to degrade polycyclic aromatic hydrocarbons and/or bioremediation of contaminated environments, which aid in detoxifying the soil and maintaining its quality [76,77]. Sb stress +2.5 % BC mainly affected *Brevundimonas* (10.3 %), *Sphyngopixis* (10.2 %), *Stenotrophomonas* (5.8 %) and *Luteimonas* (3.0 %), *Devosia* (2.0 %). Among these, *Sphyngopixis*, *Luteimonas*, *Stenotrophomonas* and *Devosia* are known for their roles in bioremediation

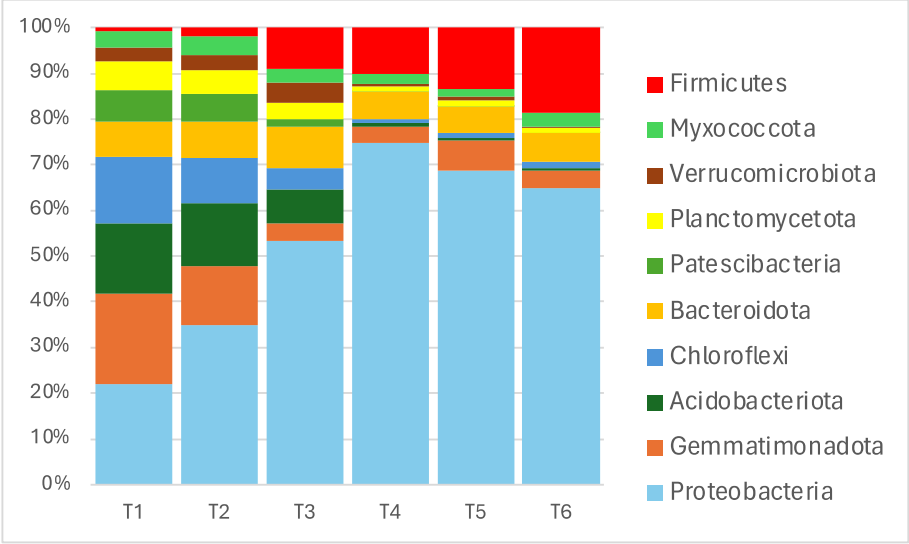

Fig. 3. Relative abundance of soil microbial community at the phylum level under different biochar treatments. T1, Ctrl; T2, Sb stress; T3, Sb stress +1 % BC; T4, Sb stress +2.5 % BC; T5, Sb stress +1 % BC + BI; T6, Sb stress +2.5 % BC + BI.

[78–81]. Sb stress +1 % BC + BI positively influenced *Stenotrophomonas* (2.4 %), *Brevundimonas* (5.3 %), *Luteimonas* (2.9 %), *Sphingopyxis* (2.3 %) and *Paenibacillus* (1.9 %). Among these *Stenotrophomonas*, *Luteimonas*, *Sphingopyxis* and *Paenibacillus* are involved in bioremediation and resistance to heavy metal [82,83]. Finally, Sb stress +2.5 % BC + BI mainly positively influenced *Luteimonas* (16 %), *Pseudoxanthomonas* (10.5 %), *Sedimentibacter* (6.3 %), *Azospirillum* (2.2 %) and *Sphyngopyxis* (1.9 %) of which *Luteimonas*, *Pseudoxanthomonas, Sedimenti*bacter and *Sphyngopyxis* are involved in bioremediation [84,85]. Focusing specifically on the *Bacillus* genus, treatments with BC alone or in combination with *B. subtilis* 1.2172 led to a statistically significant increase in its abundance. While the percentage was 0.03 % in control and 0.09 % in SB stress, it increased to 0.12 %, 1.09 %, 1.09 %, and 0.48 % in the 1 % BC, 2.5 % BC, 1 % BC + BI, and 2.5 % BC + BI treatments, respectively (Table S3).

The description of all genera present in the tested conditions is shown in Table S3 and S4. In summary, biochar, whether applied alone or combined with *B. subtilis* 1.2172, positively influenced genera primarily associated with adaptation to polluted environments. By reducing the toxicity of pollutants in soil, bacteria potentially useful for bioremediation enhance soil quality and support sustainable plant growth. The contribution to overall ecosystem health is supported by the plant growth parameters in the presence of Sb stress + BC or Sb stress + BC + BI. Considering all the replicates, discrepancies between T2R1 and the other replicates (T2R2 and T2R3) have been observed. This deviant behavior may be attributed to different technical or ecological reasons; therefore, sample T2R1 can be considered an outlier (Fig. 3 and Fig. 4).

3.4.3. Bacterial beta diversity

Beta dispersion of the bacterial community revealed that samples had the same centroid (PERMDISP: $p = 0.37$, $F = 1, 18$). NMDS was performed on the soil bacterial community in the different treatments; two principal factors were extracted (Fig. 5). NMDS had a stress value of 0.02, indicating a good fit. NMDS shows that the microbial community structure of the same treatment has a high degree of similarity, and the distance between treatments is close (Permanova p-value <0.001) (Table S5).

4. Discussion

A range of remediation materials have been investigated for their effectiveness in mitigating different metalloids contamination in agricultural soils, including apatite, organic amendments, functional microorganisms, and biochar. Among these, biochar and functional microorganisms have recently attracted significant interest due to their potential in reducing metalloid concentrations in soil. Notably, the immobilization of functional microorganisms such as *B. subtilis* onto biochar has been shown to modulate their release kinetics, thereby enhancing their stability and remediation efficiency. In addition to its bioremediation capabilities, *B. subtilis* is known to improve the microbial composition of the rhizosphere, enhance soil metabolic activity, and decrease the bioavailability of metalloids. These effects collectively contribute to a reduction in the uptake and accumulation of metalloids in crops [45]. Biochar and the associated BI determined univocal benefits under many viewpoints to the rice plant seriously impaired by Sb stress. Reduction of EL in *Poaceae* species growing on a mine ore substrate supplemented with BC has already been shown [86]. However, potential recovery almost to the levels of unstressed control (Table 1) has not yet been documented.

4.1. Plant morphological, physiological and biochemical activities

In photosynthetic pigments, a complete recovery of total chlorophyll content under Sb stress, comparable with *a* and *b* chlorophyll recovery in our experiment (Table 1), was obtained in maize with a higher BC dose (4 %) under lower stress level (200 mg Sb kg^{-1}) than in our experiment [7].

In osmo-regulating compounds (Table 1), the variations in TSP and FAA (partially restored by BC + BI vs. Sb stress) and proline (further enhanced by BC + BI vs. Sb stress) observed in this experiment are unprecedented in the literature. Compared to this, the BC + BI enhancement of the four antioxidant enzymes (CAT, POD, SOD and APX) (Table 1) indicates stronger plant ability to withstand the oxidative stress determined by Sb entering plant tissues, as shown in a previous work [7]. This is consistent with BC + BI lowering the two oxidative stress markers (MDA and H_2O_2; Table 2). This last effect was also shown by Zhu et al. [7]. Overall, it is perceived that BC + BI, on one side, countered Sb entry into plant tissues, which positively reflected in restored photosynthetic pigments, osmo-regulating compounds (except proline), and oxidative stress markers. On the other side, it strengthened plant defenses against oxidative stress by enhancing the specific antioxidant enzymes. Biochar might provide the habitat to microbes to function properly, and it also increases metabolites produced by microbes, which increases antioxidant activities to counter metalloid

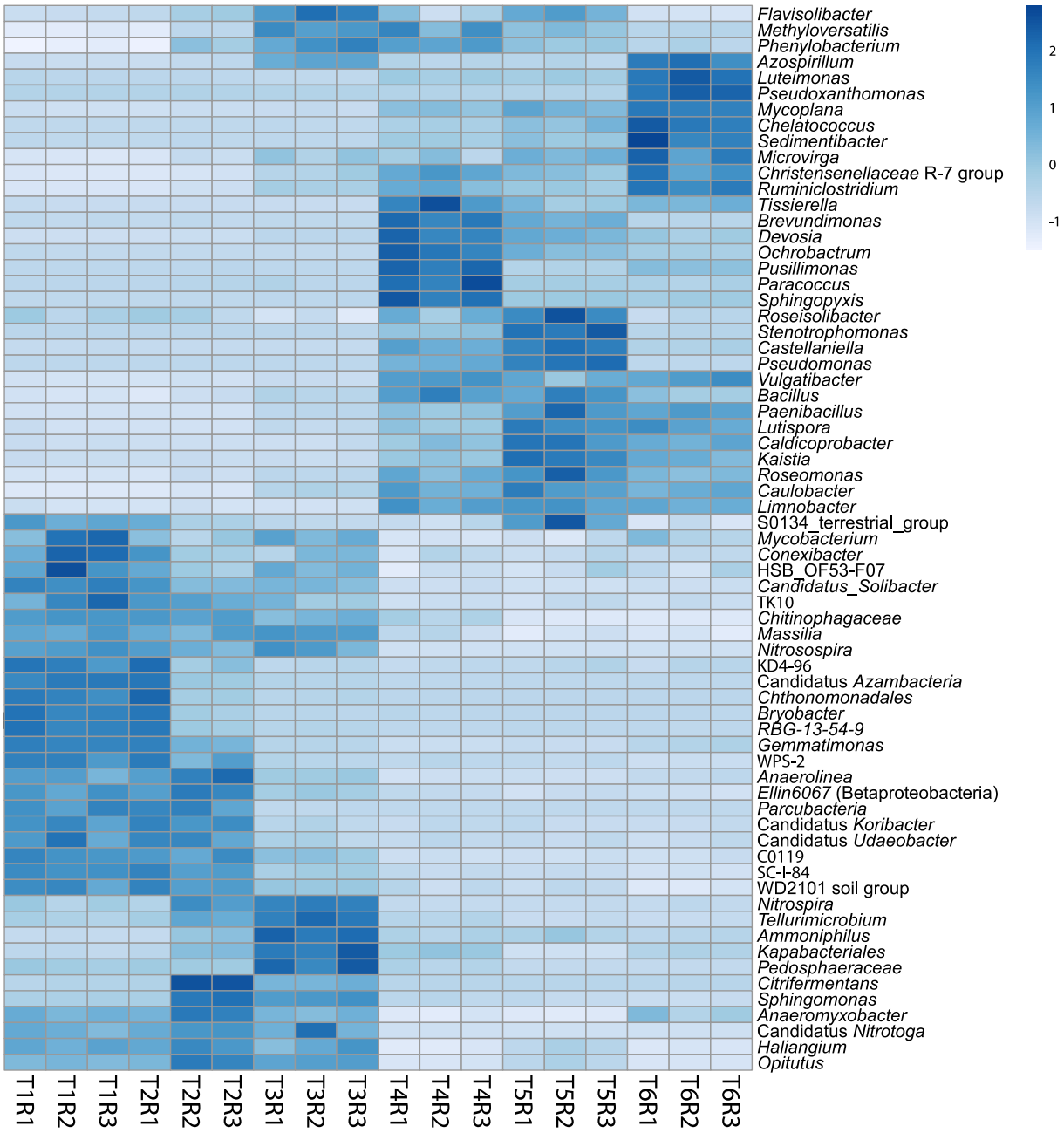

Fig. 4. Heat map showing the relative abundances and distribution of representative 16S rRNA gene tag sequences classified at the genus level. The colour code indicates the differences in the relative abundance from the mean, ranging from dark blue (positive) to light blue (negative). All treatment × replicate combinations (6 T × 3R) are shown. T1, Ctrl; T2, Sb stress; T3, Sb stress +1 % BC; T4, Sb stress +2.5 % BC; T5, Sb stress +1 % BC + BI; T6, Sb stress +2.5 % BC + BI. The values of bacterial reads and related statistics are reported in Table S1 in the Supplementary Materials. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

toxicity [87].

In morphological traits, increase in shoot length with BC under Sb stress had been observed by Zand et al. [26] in wheat. However, no comparison with an unstressed control is provided by this as well as other sources. In yield traits, there is no comparison for the sizeable GY increase obtained in this experiment with maximum stress relief vs. Sb stress, nor with an unstressed control. Effects on the three yield components indicate that BC + BI contributed more to restore the functionality of the reproductive organs (number of kernels/panicle and thousand kernel weight), than the density of reproductive organs (fertile tillers/plant).

4.2. Antimony uptake and partitioning to plant organs

The contrasting effect of BC + BI on Sb concentration (strong decrease) and content (modest decrease) in plant organs (Table 3) is consistent with BC + BI ability to buffer Sb soil availability while enhancing plant growth. This means that a growth driven Sb uptake occurred, resulting in element dilution in plant organs, and in a relevant reduction (−43 %) in Sb bio-accumulation factor. It is perceived that BC + BI alleviated Sb toxicity in rice for human consumption, while the whole plant could still contribute to significant element removal in view of reducing soil pollution. This overall effect has already been observed in other potentially toxic elements [29] and, in association with titanium dioxide nanoparticles, also in Sb [26]. The bacterial strain *B. subtilis*

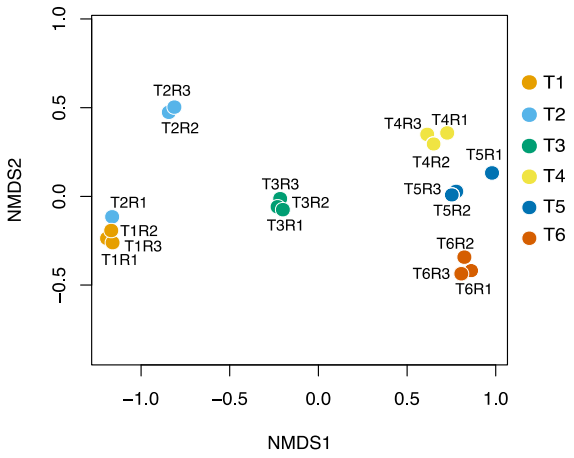

Fig. 5. Non-metric multidimensional scaling (NMDS) analysis of the soil bacterial community structure based on weighted Unifrac distance. All treatment × replicate combinations (6 T × 3R) are shown. T1, Ctrl; T2, Sb stress; T3, Sb stress +1 % BC; T4, Sb stress +2.5 % BC; T5, Sb stress +1 % BC + BI; T6, Sb stress +2.5 % BC + BI.

1.2172 showed excellent biofilm formation and produced exopolysaccharides, and secreted ACC-deaminase, which might cause Sb immobilization [88] thus reducing its availability and accumulation in plant tissues. Biochar also contained a significant amount of carbon, which increases the binding of Sb with organic matter, leading to a reduction in Sb availability and its accumulation in plant tissues [47].

4.3. Antimony bio-accessibility

In-vitro bio-accessibility is considered an effective approach to assess the health risks of metal(loid) polluted soils. Higher bio-accessibility for element uptake by organism indicates higher potential toxicity. BC and BI showed a strong impact on Sb bio-accessibility. Biochar possesses an excellent porous structure and higher surface which ensures best absorption of Sb, resulting in substantial reduction in Sb bio-accessibility [89]. Many studies have documented the appreciable potential of BC, even at lower application rates, in mitigating metal bio-accessibility in acidic soil [90,91]. BC application also facilitates Sb-soil interactions, which in turn reduce the bio-accessibility of this element. BC application influences soil pH, microbial activities and soil redox potential, which are important factors governing the availability and bio-accessibility of metals [92] and, perhaps to a lower extent, metalloids. Moreover, BC supply can improve soil physical and chemical characteristics, which can indirectly contribute to reduce Sb bio-accessibility by producing less favorable conditions for Sb uptake.

4.4. Soil traits

Soil traits, involving chemical, biological and enzymatic parameters, outlined general benefits of BC + BI vs. Sb stress (Table 4). This is consistent with supplementing a carbon rich (640 g kg^{-1}) product as BC which, at 1 % and 2.5 % supply, determined a respective 6.4 and 16 g kg^{-1}C supply to a soil whose original SOC content was 19.1 g kg^{-1}. In the rest of soil traits, improvements in soil enzymes, namely, urease with BC under Sb stress, have already been observed in previous works, as the likely consequence of the Sb immobilization determined by BC [26,27]. Antimony toxicity caused a marked reduction in nutrient availability, which could be attributed to Sb-mediated disruption in microbial activities involved in nutrient cycling and interaction of Sb with nutrients, thus reducing nutrient availability [17]. Biochar and bacterial inoculation significantly enhanced the nutrient availability. Biochar improves soil structure, soil carbon availability, and microbial activities, which ensures better nutrient uptake and availability [93,94]. The growth-promoting bacteria also promote nutrient uptake by increasing nutrient availability [95]. We also observed that the bacterial strain showed excellent ability to produce indole acetic acid, which promotes root growth and increases nutrient uptake [94]. Antimony toxicity significantly decreased the soil enzymes activity, possibly by inhibiting the bacterial and fungal growth involved in urease and catalase production [96] and decreasing nutrients availability, which play a crucial role in soil enzymes activity. Biochar and bacterial inoculation significantly enhanced the soil enzyme activity by increasing soil nutrients and carbon availability and reducing Sb availability, which improves microbial activity and maintains better soil enzyme activity [97].

4.5. Microbial applications

Applying microorganisms in agriculture for plant cultivation in heavy metal-contaminated soil could be a promising tool [98]. Strains belonging to *Bacillus* spp. have shown promising results against cadmium and lead stresses [99]. However, their potential against Sb toxicity has not been explored. In the present study, it has been shown that in *B. subtilis* 1.2172 growth, varying concentrations of Sb stimulate biofilm formation (Fig. 1). This finding aligns with the significant production of *exo*-polysaccharide by *B. subtilis* 1.2172. This production, which is positively associated with Sb concentration (Table 3), is crucial for biofilm formation and production, and can be an important factor that underscores the capability of *B. subtilis* 1.2172 to thrive in Sb-contaminated soil. Furthermore, *B. subtilis* 1.2172 has been demonstrated to potentially improve plant growth by producing IAA, which is a growth hormone, siderophores, which can protect plants from pathogens and their virulence factors, and ACC deaminase, which lowers ethylene levels in and around roots, promoting their growth and elongation as in other PGPR [99]. In this study, *B. subtilis* 1.2172 inoculation was employed to enhance BC positive impact on soil and rice plant growth. SEM analysis of BC either alone or inoculated with *B. subtilis* 1.2172 revealed that the inoculum produced a biofilm on the BC particles (Fig. S2), promoting extensive bacterial colonization that can enhance overall functionalities and improve performance.

4.6. Soil bacterial community richness, composition and diversity

Considering the effect of biochar and bacterial inoculum combined with Sb stress on the soil bacterial community in the rhizosphere where rice plants were grown, it was shown that different treatments directly affect bacterial microbiota composition. The analysis based on weighted Unifrac distance indicates that different concentrations of BC and BI in Sb stress conditions significantly impact the structure and abundance of microbial communities, with a higher similarity in the bacterial community structure of the experimental treatments Sb stress +2.5 % (T4), Sb stress +1 % BC + BI (T5), and Sb stress +2.5 % BC + BI (T6) (Fig. 3).

Lehmann et al. [100] evidenced that BC driven modifications in soil properties can influence the overall condition of the system, impacting both abiotic factors (such as available carbon, nutrients, pH, toxic substances, and water content) and biotic factors (such as alterations in habitat leading to shifts in community composition and structure).

An increased abundance of the *Bacillus* genus vs. the control and Sb stress treatments was observed in treatments with BC alone or combined with *B. subtilis* 1.2172. Due to the pivotal role of the *Bacillus* genus as a PGPR, known for supporting plant development and enhancing tolerance to environmental stressors, the enrichment of *Bacillus* in the rhizosphere represents a promising strategy for protection from metalloids and promoting sustainable agricultural forms contributing to global food security [92,101]. Moreover, microbiota soil analysis showed that BC alone and supplemented with BI significantly changed microbial community structure at the phyla, families, and genera levels. For example, the genera *Paenibacillus, Luteimonas*, and *Caulobacter* increase in the specific treatments (Fig. 4).

In our study, the relative abundance of *Paenibacillus* increased

significantly in treatments with 1.0–2.5 % BC and 1.0–2.5 % BC + BI (Table S3). Recent studies revealed that many strains belonging to *Paenibacillus* species showed multiple plant growth-promoting effects (Table S4) that improved plant vegetative and reproductive growth [102]. Many members of the genus *Luteimonas* are strongly associated with the plant rhizosphere environment, with some species able to degrade complex organic compounds, resulting in increased nitrogen or phosphorus uptake by plants [103,104]. Strains belonging to *Caulobacter* species improves growth and heavy metal resistance, mitigating heavy metal accumulation in terrestrial ecosystems. Furthermore, *Caulobacter* is suggested as a model organism for plant microbiome studies, alongside its use in biotechnological applications in plant growth promotion and heavy metal resistance [105].

Finally, the genus *Parapedobacter* increased only in treatments with Sb stress added with BC and BI (Fig. 4). Recent studies showed that *Parapedobacter* can produce bio emulsifiers and siderophores, making it an intriguing candidate for heavy metal removal from contaminated soils [106]. Bio emulsifiers can promote metalloid extraction from contaminated soil through different mechanisms, such as mobilization by ionic exchange and the interaction between the biosurfactant monomers and metalloids, followed by their incorporation and stabilization in the center of the formed micelles [107]. It is widely recognized that bacterial siderophores, known for their ability to bind iron, can also chelate many metalloids, reducing their bioavailability and toxicity to plants and other organisms [108,109]. The finding of beneficial taxa that increase in the rhizosphere microbiota of rice after treatment with Sb stress added with biochar (BC) and *B. subtilis* (BI) supports the importance of such treatments for helping to improve plant growth in Sb-polluted soil.

5. Conclusions

Biochar and bacterial inoculated biochar proved valuable partners in mitigating Sb toxicity in rice. Biochar + bacterial inoculation appreciably increased the rice physiological functioning and yield. This was linked with improved antioxidant defense, osmoprotectant production, reduced oxidative damage and Sb availability, increased soil enzyme activity, soil carbon and nutrients availability, and diversity and abundance of favorable microbes. Although we did not directly measure the adsorption/fixation of *Bacillus subtilis* by biochar or its release efficiency, our results show that the abundance of the *Bacillus* genus increased to varying degrees across treatments, suggesting a potential effect of the added *B. subtilis*. This is generally demonstrated in most plant traits, by the treatment involving maximum stress relief (2.5 % BC + BI) which closed an average three-quarters of the gap between Sb stress and unstressed control. On one side, this points out that prevention of contamination is a fundamental precautionary measure to avoid loss of soil fertility affecting agricultural production on a large scale. On the other side, a feasible mitigation strategy for contaminated soils is outlined in this work. We used ICP-MS technique to determine the Sb concentration in plant tissues. This technique provides many benefits, including exceptional sensitivity with detection limits, rapid and simultaneous analysis of different elements, and greater accuracy. This is a short term study conducted to explore the impacts of BC and bacterial inoculation in mitigating Sb toxicity. Future, studies should explore the long-term effect of the current technique in improving soil health, plant productivity and mitigating antimony toxicity. Looking ahead, the study of the microbial consortium present in polluted soil is seen a key point especially as it concerns plant growth. Investigating bacteria that thrive in stressful conditions, such as the presence of potentially toxic elements, could provide valuable insights into the synergistic effects of combining bacterial bioremediation with plant-based approaches.

CRediT authorship contribution statement

Muhammad Umair Hassan: Writing – original draft. **Lorenzo Barbanti:** Writing – original draft. **Luigimaria Borruso:** Writing – original draft. **Paola Mattarelli:** Writing – review & editing. **Monica Marianna Modesto:** Writing – review & editing. **Huang Guoqin:** Supervision, Funding acquisition. **Duan Renyan:** Writing – review & editing. **Haiying Tang:** Writing – review & editing. **Faizah Amer Althani:** Writing – review & editing.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.enceco.2025.08.002.

Data availability

Data will be made available on request.

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