



# Alleviating Cr(VI) stress in horse gram (*Macrotyloma uniflorum* Var. Madhu) by native Cr-tolerant nodule endophytes isolated from contaminated site of Sukinda

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## Abstract

Sukinda chromite mine of Odisha is a heavily polluted site, generating huge overburden dumps. The present experiment was designed to evaluate the potential of two native nodule endophytic bacterial strains, viz. *Bacillus aryabhattai* AS03 (MT645244) and *Rhizobium pusense* AS05 (MT645243), isolated from contaminated sites to be considered remediation tool to minimize the effect of Cr toxicity on *Macrotyloma uniflorum* var. Madhu. The two nodule endophytic bacterial strains AS03 and AS05 exhibited tolerance to 1800 and 3000 ppm of Cr(VI) respectively in vitro when cultured alone. AAS analysis confirmed higher accumulation of Cr(VI) in roots and less accumulation in shoots which is dose-specific (bio-inoculant) either treated alone or combined. Complete absence of Cr accumulation approximately 99% in shoots of *Macrotyloma* was observed owing to synergistic effect of both the strains (biochar-based formulation). This study also suggests increased shoot and root length, nodule nos., and leghemoglobin content of the plant at 60 days indicating the plant growth-promoting effects of both the strains. ROS and antioxidant enzymes of the plant recorded decreasing trend in inoculated plants. However, a significant increment in transpiration rate, total photosynthetic rate, intracellular CO<sub>2</sub> conc., and stomatal conductance in leaves was observed owing to dual inoculation. Our findings corroborate the supremacy of synergistic effect of both the strains applied in the form of biochar-based biofertilizer in enhancing growth and tolerance index of *M. uniflorum* cultivated in Cr(VI)-stressed soil. This investigation depicts the efficiency of the two nodule bacteria as a mixed inoculant to alleviate Cr toxicity and making the seeds safe for consumption.

**Keywords** Biochar amendment · Bio-inoculation · Cr toxicity · Nodule endophytes · *Rhizobium* · ROS levels

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Shilpee Dhali and Madhusmita Pradhan contributed equally to this work.

## Highlights

- Isolation of Cr(VI)-tolerant nodule endophytes from contaminated site of Sukinda.
- Dual bacterial inoculation resulted in no accumulation of Cr in shoots at 60 days.
- Nodule endophytes benefited the crop with respect to morpho-physicochemical characteristics.
- Plants with biochar formulation showed 94.97% tolerance index under Cr toxicity.

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## Introduction

India stands fourth (4th) in chromite mineral production, and the state Odisha is the sole producer of chromite mineral production (99.99 %) (Ministry of Mines 2019). According to report published in Economic Survey of Odisha (2019), chromite mining area of the state is approximately 5829.30 ha and a major part is contributed from Sukinda, Jajpur District (Govt. of Odisha 2019). Hexavalent chromium (Cr(VI)) toxicity in soil induces severe damages to the plant. Cr(VI) is reduced to other oxidation states after entering the plant cell and attacks cellular proteins, DNA, and membrane proteins ultimately leading to physiological and morphological stress in various crops (Stambulska et al. 2018; Panda et al. 2020; Dhali et al. 2020).

Leguminous crops known for their contribution to the biological nitrogen fixation processes are often affected by Cr toxicity. Previous studies reported that nitrogenase enzymes were inactivated by Cr(VI) toxicity leading to deterioration of nodule activity and reduction of nitrogen fixation (Sangwan et al. 2014). The scope of nodule bacteria with resistance to Cr toxicity is undoubtedly the important factor to be exercised. According to past studies, Cr-tolerant nodule bacterial strains possess the efficacy of accumulating Cr in nodules while making plants to be less exposed to toxic effects (Kong et al. 2015). The protective way out of nodule bacteria for the plants to mitigate toxicity besides maintaining yield consists of possible reduction of hexavalent Cr to trivalent, binding with other compounds and elimination, upregulation of antioxidant defense system (Narayani and Shetty 2013; Stambulska et al. 2018).

Among legumes, pulse crops constitute an important category which assimilate ammoniacal nitrogen with the help of nodule symbionts, maintain soil fertility by facilitating nutrient mineralization, and also provide high protein food to the growing population (Stagnari et al. 2017; Das et al. 2020). In developing countries where majority being small and marginal farmers along with increasing population, pulse crops must be prioritized to boost crop health and yield in contaminated sites. In this context, extensive research should be carried out in the pursuit of novel Cr-tolerant nodule endophytes to safeguard crop and soil health.

The present investigation is focused on horse gram (*Macrotyloma uniflorum* cv. Madhu) which is of utmost nutritional and medicinal importance. The edible seed of this underexplored crop is known to have phenolic acids, flavonoids, and antioxidants and can benefit in controlling fat cells, diabetes, blood cholesterol levels, and diuretic and urolithiatic activities (Bhartiya et al. 2015; Patel and Acharya 2020). In this study, Cr(VI)-tolerant nodule endophytes were isolated from *Macrotyloma uniflorum* var. Madhu crop grown on chromium (VI)-toxic overburden soil, collected from mine area of Sukinda, Odisha, and their possible application to mitigate the toxic effect of Cr(VI) on the plant.

## Materials and methods

### Study site and pot experiment

Chromium-rich overburden soil (OBS) samples (approximately 75 kg) were collected from 30 cm depth of the experimental site from chromite mining area (South Kaliapani location), Sukinda (latitude 21° 1' to 21° 4' N and longitude 85° 45' to 85° 48' E), Jajpur District, Odisha (Fig. 1). Soil physicochemical analyses, viz, soil texture (USDA soil taxonomy) following Bouyoucos method, pH (soil to water as 1:2.5 ratio), cation exchange capacity (Jackson 1975), organic carbon, exchangeable Ca and Mg (Page et al. 1982), available N (Subbaiah and Asija 1956), P<sub>2</sub>O<sub>5</sub> (Bray and Kurtz 1945), and K<sub>2</sub>O (Jackson 1975), were carried out in Soil Chemistry Laboratory of Department of Soil Science and Agricultural Chemistry, OUAT, Bhubaneswar, to characterize the initial soil samples. Digestion of soil samples was carried out with diacid (HNO<sub>3</sub> to HCl—1:3 ratio) and filtered. Available Cr concentration of samples was determined using Atomic Absorption Spectrophotometer (AAAnalyst 200; Perkin Elmer, USA).

Certified seeds of *Macrotyloma uniflorum* var. Madhu were obtained from OUAT, Bhubaneswar. Seed sterilization was carried out dipping them in 1% sodium hypochlorite (NaOCl) for 6 min. Seeds were rinsed 6–7 times repeatedly for 15–20 mins followed by drying under sterile air stream for about 8–10 h. Seeds were soaked in sterile distilled water for 12 h before sowing. Five (05) sets of pots with pot mixture (OBS to fine sand to vermicompost—3:2:1) were prepared, and the presoaked seeds were sown. Due care and maintenance were followed until 110 days.

### Isolation of nodule endophytes from *Macrotyloma uniflorum* var. Madhu

Plants (60 days) were uprooted carefully and washed properly. Isolation of endophytes from root nodules of horse gram plants was carried out following methods documented by Vincent (1970). Nodules were separated from the roots and washed properly in tap water to remove all the impurities from the surface. After proper cleaning, they were dipped in 0.1% NaOCl solution for 30 s followed by repeated wash with sterile distilled water. Furthermore, the nodules were crushed with sterile distilled water and inoculated on yeast extract mannitol agar (YEMA) containing Congo Red (incubation at 28 ± 2 °C for 48 h). The endophytic bacterial cultures were maintained on Luria-Bertani Agar (LA) medium for further experiments.

### Minimum inhibitory concentration (MIC) of Cr(VI)

The endophytic bacteria (no. 06) were streaked on LA medium supplemented with different concentrations of potassium

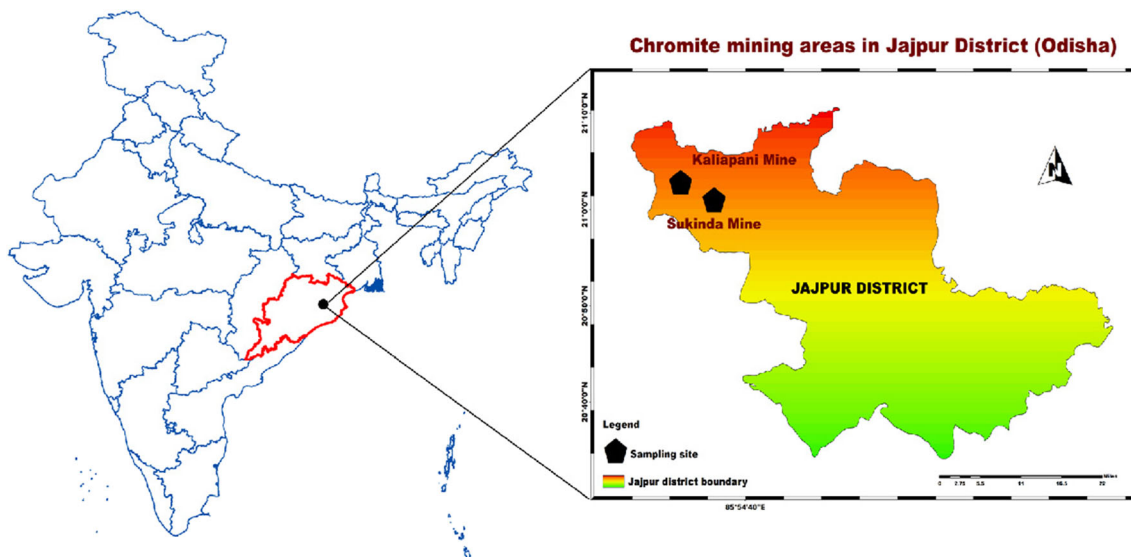


Fig. 1 Map of chromite mine area Sukinda, Jajpur District in the state of Odisha, India

dichromate ( $K_2Cr_2O_7$ ) (200, 250, 300, 350, 400, 500, 600, 750, 900, 1200, 1500, 1800, 1900, 2000, 2300, 2600, 2900, and 3000  $mg L^{-1}$ ) to screen their level of tolerance to the heavy metal in vitro. The cultured plates were kept in an incubator at  $30 \pm 2 \text{ }^\circ C$  for 48 h (Cappuccino and Sherman 2013).

**Molecular identification of Cr(VI)-tolerant nodule endophytic bacteria**

Out of six (06) isolates, two (strains AS03, AS05) were further characterized for 16SrRNA gene sequencing for molecular identification and phylogeny. The 16S rRNA gene was amplified in PCR using 27F (primer1) (5'-AGAG

TTTGATCCTGGCTCAG-3') and 1492R (primer2) (5'-TACGGTTACCTTGTTACGACTT-3') (Yushmanov and Chumakov 1988). The PCR products were aligned with other related sequences available in NCBI database using multiple sequence alignment software ClustalW2, and dendrogram was constructed.

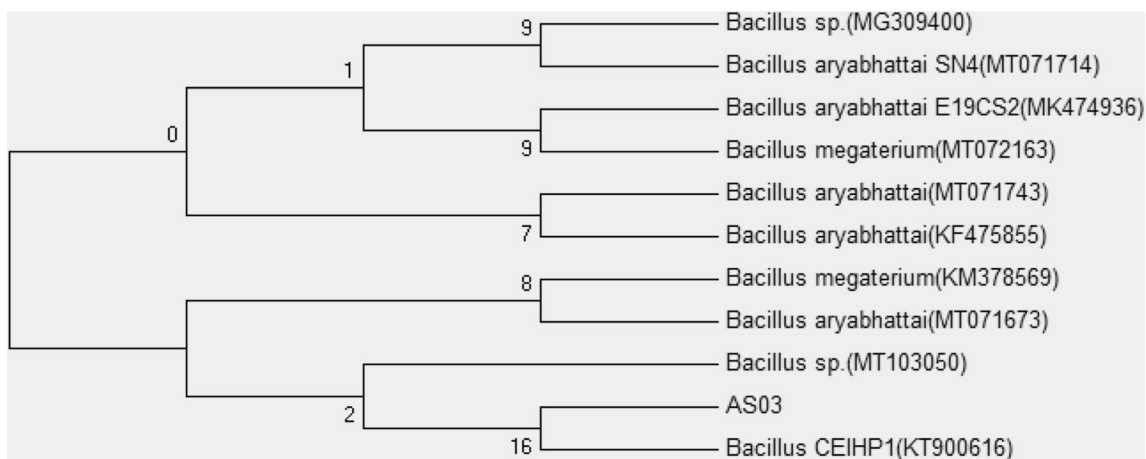
**Pot culture experiments**

Two (02) sets of pot culture studies were conducted during January 2020 at the Department of Botany, Utkal University. Both the studies were executed under Cr stress in presence and absence of Cr-tolerant nodule endophytes. The treatment details for the pot culture experiment 1 were  $C_0$ \_control,  $C_1$ —

Table 1 Tolerance of nodule endophytes to different Cr(VI) concentrations on Luria-Bertani Agar (LA) medium

Chromium concentrations ( $mg L^{-1}$ )	AS01	AS02	AS03	AS04	AS05	AS06
200	+++	++	+++	++	+++	-
250	+++	++	+++	++	+++	-
300	+++	++	+++	++	+++	-
350	+++	++	+++	++	+++	-
400	+++	+	+++	+	+++	-
500	++	+	+++	+	+++	-
600	++	+	+++	+	+++	-
750	+	+	+++	+	+++	-
900	-	-	+++	-	+++	-
1200	-	-	+++	-	+++	-
1500	-	-	++	-	+++	-
1800	-	-	+	-	+++	-
1900	-	-	-	-	+++	-
2000	-	-	-	-	++	-
2300	-	-	-	-	++	-
2600	-	-	-	-	++	-
2900	-	-	-	-	+	-
3000	-	-	-	-	+	-

+++ maximum growth, ++ moderate growth, + less growth, - no growth



**Fig. 2** Phylogenetic tree of *Bacillus aryabhatai* AS03 (MT645244)

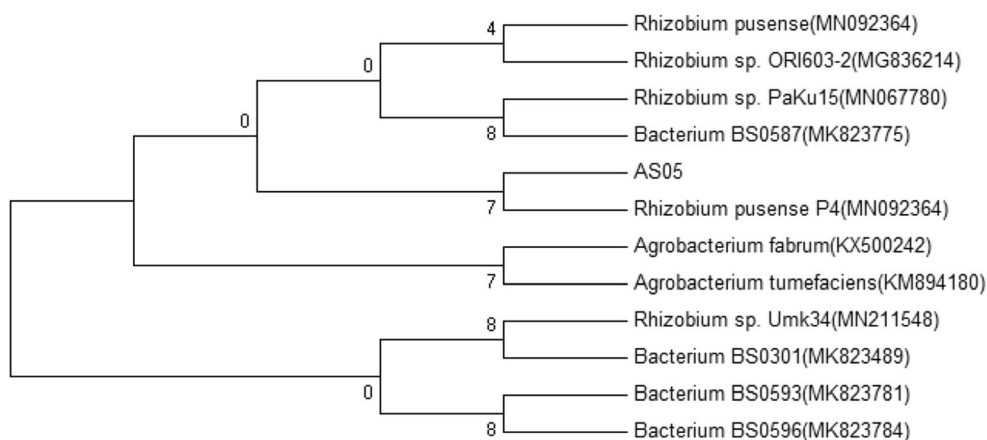
Cr(VI) 15 mg kg<sup>-1</sup>, C<sub>2</sub>—Cr(VI) 30 mg kg<sup>-1</sup>, C<sub>3</sub>—Cr(VI) 60 mg kg<sup>-1</sup>, and C<sub>4</sub>—Cr(VI) 90 mg kg<sup>-1</sup>. Additionally, treatments for pot culture experiment 2 were T<sub>1</sub>—control, T<sub>2</sub>—Cr(VI) 90 mg kg<sup>-1</sup> + AS03, T<sub>3</sub>—Cr(VI) 90 mg kg<sup>-1</sup> + AS05, T<sub>4</sub>—Cr(VI) 90 mg kg<sup>-1</sup> + no seed priming + AS03 + AS05, T<sub>5</sub>—Cr(VI) 90 mg kg<sup>-1</sup> + hydropriming + AS03 + AS05, T<sub>6</sub>—Cr(VI) 90 mg kg<sup>-1</sup> + biochar-based inoculum (AS03), T<sub>7</sub>—Cr(VI) 90 mg kg<sup>-1</sup> + biochar-based inoculum (AS05), and T<sub>8</sub>—Cr(VI) 90 mg kg<sup>-1</sup> + biochar-based inoculum (AS03 + AS05). Proper care and maintenance were followed until 110 days for growth of plants in the treated pots until maturity and then harvested.

### Bio-inoculation

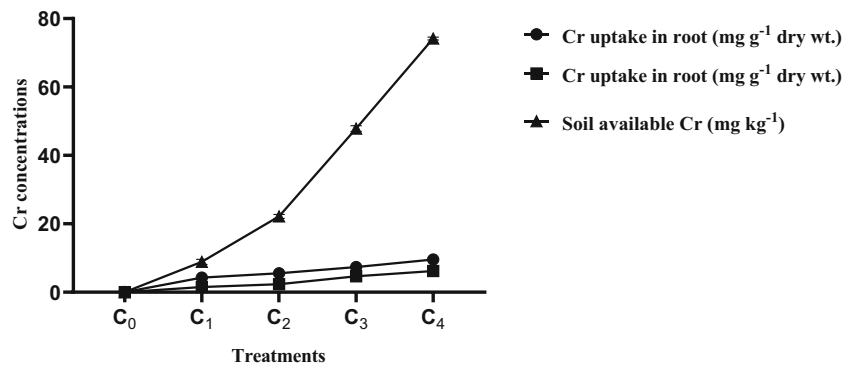
Horse gram (*Macrotyloma uniflorum* var. Madhu) seed surfaces were disinfected with 1% sodium hypochlorite for 6 min, then repeatedly (6 times) rinsed with sterile distilled water for 15–20 min and dried overnight in the presence of sterile air. Required quantities of the seeds were soaked in sterile distilled water for 6 h before sowing (hydropriming). The

endophytic strains (AS03 and AS05) were inoculated to 100 mL LB and incubated at 28 ± 2 °C and 120 rpm for 48 h to achieve cell count of 10<sup>8</sup> to 10<sup>9</sup> colony forming units (CFU) mL<sup>-1</sup> of broth. The broths were centrifuged at 12,000 rpm; the resultant pellets were washed with 0.1 M phosphate buffer (pH—7.0). Entire content was then dissolved in phosphate buffer (cell count 10<sup>8</sup> CFU mL<sup>-1</sup>). According to the requirement of the experiment, the seeds (hydroprimed and non-primed) were soaked in phosphate buffer for 4–6 h. Approximately 3 mL phosphate buffer was used for each seeds (Dey et al. 2004; Pradhan et al. 2017). Solid carrier formulation for biofertilizer was composed of rice straw biochar: fine sand in 1:1 ratio. Each of the material was sterilized in autoclave followed by UV irradiation. Biochar of rice straw was prepared from pyrolysis under very little oxygen concentration at 550–650 °C over 1-h time period. The properties of biochar were (pH—8.1, C—83.6%, N—1.22%, WHC—75.63%) determined. Six hundred eighty milliliters of cultured LB medium was mixed per 1 kg of carrier formulation. Approximately 50 g of biofertilizer formulation was applied per 1-kg soil following the treatment schedules.

**Fig. 3** Phylogenetic tree of *Rhizobium pusense* AS05 (MT645243)



**Fig. 4** Effect of Cr(VI) on soil avail Cr and total Cr contents in roots and shoots of *M. uniflorum* var. Madhu at 60 days



**Plant growth attributes**

Sixty (60)-day-old plants from both the pot culture experiments were subjected to record the root and shoot biomass (length and dry weight), number of nodules, and dry weight of nodules. Plant samples were air-dried followed by drying in an oven at 75 °C until constant weights were obtained.

**Estimation of chlorophyll, protein, proline, and leghemoglobin content**

Total chlorophyll concentration of leaves was calculated with equation given in Porra et al. (1989). Soluble protein, total carbohydrates, and reducing sugar were estimated following methods described by Bradford (1976), Sadasivam and Manickam (2008), and Somogyi (1952), respectively. Total phenolic content (Xu and Chang 2008), flavonoids (Xu and Chang 2007), proline (Bates et al. 1973), and leghemoglobin contents (Wilson and Reisenauer 1963) were recorded following standardized protocols.

**Biochemical and antioxidant parameters**

Extracellular hydroxyl radicals (Halliwell et al. 1987), superoxide radical (Jiang and Zhang 2001), hydrogen peroxide (Velikova et al. 2000), and lipid peroxidation in terms of malondialdehyde (Zhou and Leul 1999) of leaves and roots were estimated at 60 days of crop growth. The antioxidant

enzymes, viz, catalase (Aebi 1984), peroxidase (Zhou and Leul 1999), superoxide dismutase (Bergmeyer et al. 1974), ascorbate peroxidase (Nakano and Asada 1981), guaiacol peroxidase (Zhou and Leul 1999), reduced glutathione, oxidized glutathione, and total glutathione (Law et al. 1983), were also observed at 60 days.

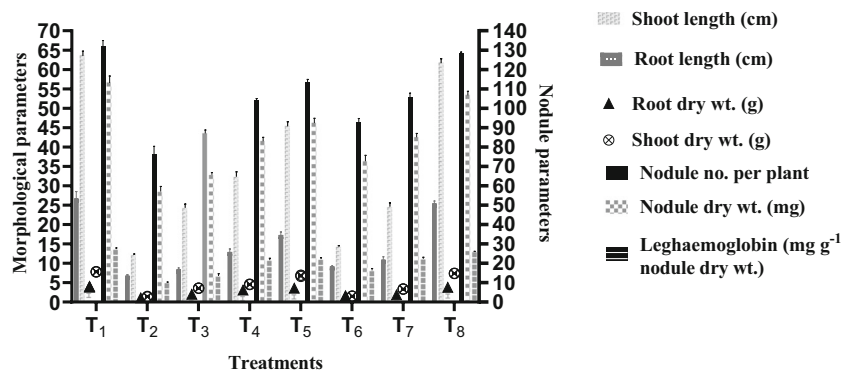
**Measurement of photosynthetic activities**

Different photosynthetic parameters such as the rate of photosynthesis, photosynthetic yield, intracellular CO<sub>2</sub> conc., CO<sub>2</sub> release, stomatal conductance, and the rate of transpiration were recorded using an infrared gas analyzer (IRGA; LICOR, <http://www.licor.com>) on sunny days in between 11:00 am to 12:00 noon.

**Available Cr in soil, total Cr accumulated in plant, and Cr tolerance indices**

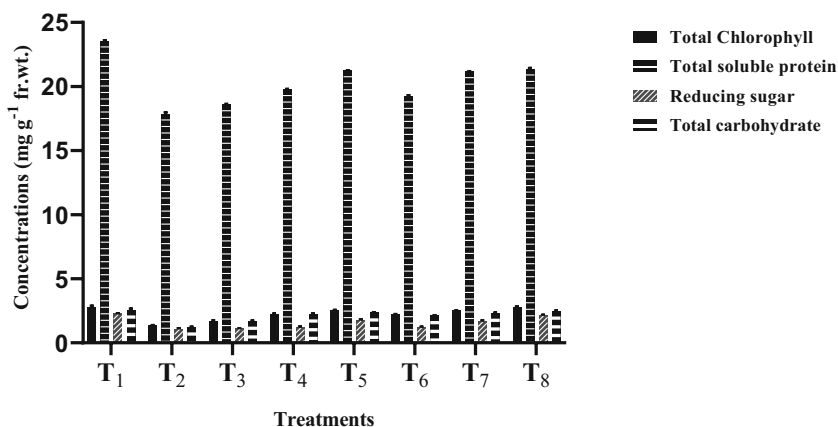
Soil samples were dried under shade, grounded to fine powder, sieved, and acid-digested (HNO<sub>3</sub> to HCl—1:3). The content was then filtered and analyzed for available Cr in an Atomic Absorption Spectrophotometer (AAAnalyst 200 Perkin Elmer, USA). Total Cr concentrations in root and shoot samples are estimated at 60 d after sowing of seeds. Digestion of plant samples with diacid (HNO<sub>3</sub> to HClO<sub>4</sub>—10:1) were carried out. Total Cr contents of plant samples were determined in an Atomic Absorption Spectrophotometer

**Fig. 5** Effect of Cr(VI) and bio-inoculation on plant morphological parameters and leghemoglobin content of *M. uniflorum* var. Madhu at 60 days





**Fig. 6** Effect of Cr(VI) on total chlorophyll, total soluble protein, reducing sugar, and total carbohydrate concentrations in *M. uniflorum* var. Madhu at 60 days



(AAAnalyst 200 Perkin Elmer, USA) (Mohanty and Patra 2012). Bio-concentration factor (BCF), total accumulation rate (TAR), and transportation index (Ti) were calculated (Ghosh and Singh 2005; Zurayk et al. 2002). The following are the formulae:

$$\text{BCF} = \text{Cr conc.in plant (mg kg}^{-1}\text{)}/\text{Cr in soil (mg kg}^{-1}\text{)}$$

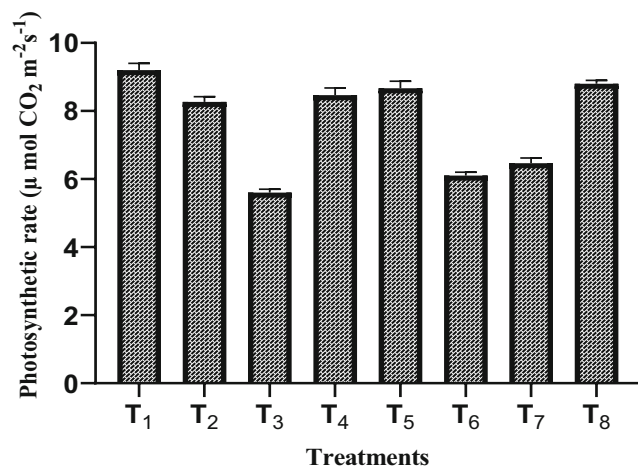
$$\text{Ti} = \text{Cr conc.in shoot (mg kg}^{-1}\text{)}/\text{Cr conc.in root (mg kg}^{-1}\text{)} \times 100$$

$$\text{TI} = \text{Dry weight of treated plants}/\text{Dry weight of control plants} \times 100$$

$$\text{TAR} = \text{Cr in Shoot} \times \text{Shoot biomass} + \text{Cr in Root} \times \text{Root biomass}/[(\text{Shoot biomass} + \text{Root biomass}) \times \text{Days of growth}]$$

## Statistical analysis

Software R version 4.0.2 is utilized for statistical analysis using the kit “agricolae” with DMRT (at  $p < 0.05$ ). Means with same alphabet are not significantly dissimilar. Data



**Fig. 7** Effect of Cr(VI) on photosynthetic performances of *M. uniflorum* var. Madhu at 60 days

presented in tables are the mean value  $\pm$  standard error of mean (SEM). Graphs were plotted with GraphPad Prism 8.2.1 (GraphPad Software, Inc., San Diego, CA). Data represents the mean value  $\pm$  standard deviation (SD).

## Results and discussion

### Isolation of nodule endophytes from *Macrotyloma uniflorum* var. Madhu grown in Cr(VI)-rich OBS

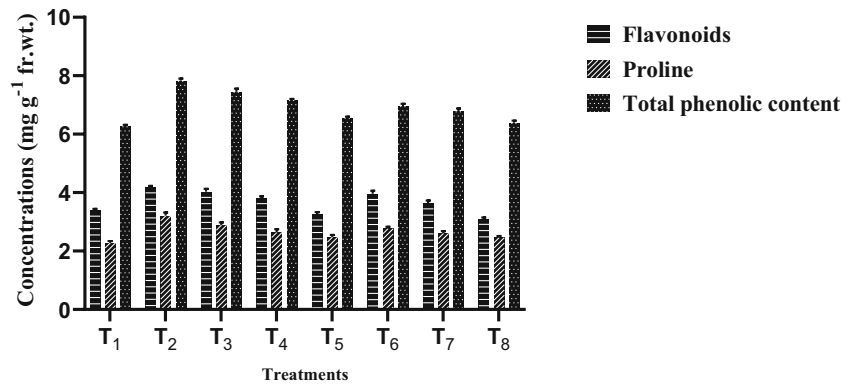
Cr (VI) is one of the major heavy metal pollutants, deteriorating soil fertility and crop health. Soil microorganisms with dual ability to tolerate the toxic effects of Cr as well as to exhibit plant growth-promoting traits can definitely be an environment-friendly and cost-effective way out for the mitigation of challenges in Cr-stressed soil. In the present investigation, the OBS was used to isolate Cr-tolerant nodule endophytes from *Macrotyloma uniflorum* var. Madhu plants.

A pot experiment was carried out with OBS collected from chromite mine area of Sukinda, Odisha, to isolate effective Cr(VI)-tolerant bacteria from nodules of *M. uniflorum*. The OBS was clay loam (sand—38.88%, silt—29.12%, and clay—32.00%) in texture with 41% water holding capacity, acidic pH (5.40), low organic carbon (0.43 %), and low in available nutrients (N, P, and K) (73.22, 5.87, and 83.25 mg kg<sup>-1</sup>) respectively, and it high available Cr (94.56 mg kg<sup>-1</sup>), 8.92 (mg kg<sup>-1</sup>) available S, 2.58 (mg kg<sup>-1</sup>) available Fe, 0.067 (μg TPF g<sup>-1</sup> soil h<sup>-1</sup>) dehydrogenase activity, 87.23 (μg C g<sup>-1</sup> soil) microbial biomass carbon, and 7.58 (log CFU g<sup>-1</sup> dry wt. soil) total heterotrophic bacterial population.

### Minimum inhibitory concentration of Cr(VI)

*Macrotyloma* plants (60 days old) grown on OBS sample collected from Sukinda uprooted and six (06) different nodule endophytes showed a wide range of resistance pattern to Cr(VI) on LA medium (Table 1). AS03 and AS05 were found

**Fig. 8** Effect of Cr(VI) on flavonoids, proline, and total phenolic contents in *M. uniflorum* var. Madhu at 60 days



resistance to 1800 and 3000 mg L<sup>-1</sup> Cr(VI) on LA medium. The isolates AS01, AS02, and AS04 survived up to 750 mg L<sup>-1</sup> Cr(VI). However, the AS06 isolate was unable to tolerate the lowest concentration of Cr(VI), i.e., 200 mg L<sup>-1</sup>. The two isolates (AS03 and AS05) with higher resistance to Cr(VI) were selected for molecular identification and pot culture experiment. Hao et al. (2012a, b) isolated heavy metal-tolerant plant growth improving rhizobacteria from the root nodules of *Robinia pseudoacacia* grown in contaminated areas. Fan et al. (2018) conducted experiments and isolated 82 endophytic bacteria from the root nodules of *Robinia pseudoacacia* grown in Pb/Zn mining area.

**Phylogeny of Cr(VI)-tolerant nodule endophytes**

The 16S rRNA sequences of the two isolates (AS03 and AS05) were obtained by directly sequencing the PCR products. Sequence analysis of the alignment through NCBI BLAST software revealed that the sequences of AS03 and AS05 showed > 99% similarity with *Bacillus* sp. and *Rhizobium* sp., respectively. Phylogenetic tree was constructed for both AS03 and AS05 on the basis of 16SrRNA sequence similarity revealed close phylogenetic relationship with *Bacillus* sp. and *Rhizobium* sp., respectively (Figs. 2 and 3). The two nodule endophytes were identified as *Bacillus aryabhatai* strain AS03 (NCBI accession no. MT645244) and *Rhizobium pusense* strain AS05 (NCBI accession no. MT645243). In current scenario, need for the isolation and utilization of a novel

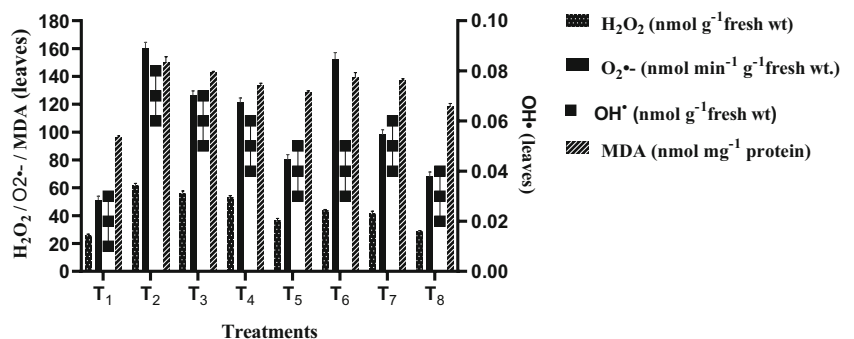
Cr-tolerant nodule endophyte is essential to decontaminate plants from the toxic effect of Cr(VI). Previous studies have reported various Cr(VI)-reducing bacteria, viz, *Pseudomonas* (Rajkumar et al. 2005), *Bacillus* (Wani et al. 2007a; Wani and Khan 2010), and *Mesorhizobium* (Wani et al. 2008) have been isolated from soils.

**Cr bioaccumulation without inoculation of nodule endophytes**

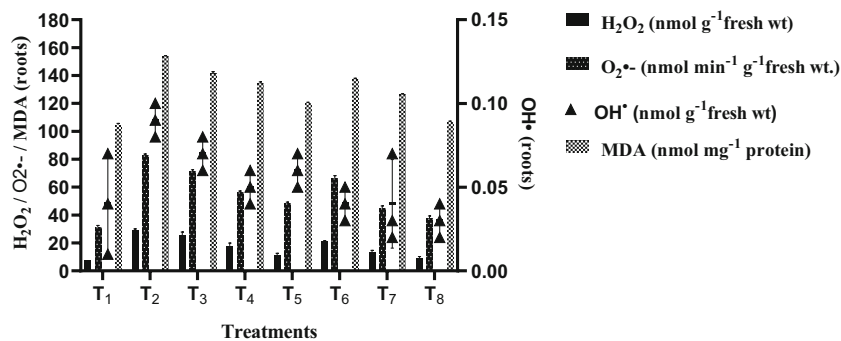
In this study, increasing doses (15, 30, 60, and 90 mg kg<sup>-1</sup>) of Cr(VI) were applied to *Macrotyloma uniflorum* to check extent of bioaccumulation in shoots and roots. The data on Cr contents in shoots, roots, and soil at 60 days of growth is summarized in Fig. 4. The total chromium (Cr) content in shoots and roots of *Macrotyloma uniflorum* cv. Madhu increased significantly with increasing doses of Cr(VI), after 60 days of growth. The highest concentration of total Cr both in shoots and roots was measured in the treatment C<sub>4</sub> (90 mg kg<sup>-1</sup> Cr<sup>6+</sup>) followed by C<sub>3</sub> (60 mg kg<sup>-1</sup> Cr<sup>6+</sup>). No traces of Cr were found in the control treatment, owing to no addition of hexavalent Cr. However, higher concentrations of Cr were observed in the roots compared to shoots of the treated plants.

In addition to bioaccumulation, we have also checked the soil available Cr at 60 days of crop growth, which revealed significantly higher amounts of Cr in treatments C<sub>2</sub> (30 mg kg<sup>-1</sup> Cr<sup>6+</sup>), C<sub>3</sub> (60 mg kg<sup>-1</sup> Cr<sup>6+</sup>), and C<sub>4</sub> (90 mg kg<sup>-1</sup> Cr<sup>6+</sup>). Furthermore, higher concentrations of soil available Cr was

**Fig. 9** Effect of Cr(VI) and bio-inoculation on reactive oxygen species (ROS) and malondialdehyde (MDA) in leaves of *M. uniflorum* var. Madhu at 60 days



**Fig. 10** Effect of Cr(VI) and bio-inoculation on reactive oxygen species (ROS) and malondialdehyde (MDA) in roots of *M. uniflorum* var. Madhu at 60 days



recorded in pots treated with C<sub>1</sub> (15 mg kg<sup>-1</sup> Cr<sup>6+</sup>) which were found to be at par with the control pots (C<sub>0</sub>). Previous research observations from Patra et al. (2018) and Dhali et al. (2020) corroborate with our findings.

Cr is not an essential plant nutrient and hence not at all crucial for crop growth and metabolic activities, and therefore, once taken up by roots, its accumulation have various toxic effects on agricultural crops (Zayed and Terry 2003; Oliveira 2012; Patra et al. 2018; Patra et al. 2020). Toxic effect of Cr can adversely affect the chlorophyll contents of leaves, derogative changes in enzyme activities, protein inactivation, damage of DNA, declining photosynthetic activity, and insufficient nutrient uptake resulting in poor plant growth (Zayed and Terry 2003).

### Cr bioaccumulation with inoculation of nodule endophytes

The 2nd pot culture experiment of horse gram was carried out with *Bacillus aryabhattai* AS03 and *Rhizobium pusense* AS05 as bacterial inoculant to mitigate hazardous effect of Cr(VI)-stressed soil.

### Effect Cr(VI)-tolerant nodule endophytes on growth of *Macrotyloma uniflorum*

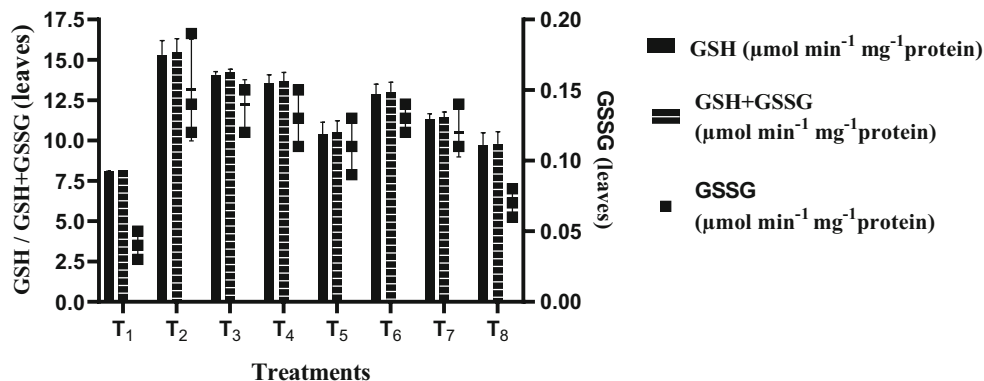
Bio-inoculation of Cr(VI)-tolerant bacterial endophytes (*B. aryabhattai* AS03 and *R. pusense* AS05) showed

promising findings in response to morphological parameters at 60 days of growth (Fig. 5). The treatment T<sub>8</sub> comprising both AS03 and AS05 applied as biochar-based biofertilizer exhibited significant increment in length and dry weight of root and shoot in comparison with treatments T<sub>6</sub> and T<sub>7</sub> (sole applications of AS03 and AS05), respectively. Biochar is a carbon-rich fine substance and can also function as a good alternative to lignite for biofertilizer formulation (Saranya et al. 2011). Besides, improving soil quality and fertility application of biochar also have the efficiency to promote immobilization of heavy metal and contaminants present in soil (Nartey and Zhao 2014).

However, plant parameters, viz, nodule no. per plant, nodule dry wt. and leghemoglobin contents, exhibited noticeable results in treatment T<sub>7</sub> (sole applications AS05) which were found to be alike with treatments T<sub>8</sub> and T<sub>5</sub>. Plant growth-promoting bacteria with the ability to immobilize Cr in root region have contributed in decreasing the effect of chromium toxicity and increasing plant biomass in Cr-toxic soil (Soni et al. 2014; Maqbool et al. 2015). According to Edulamudi et al. (2019), nodule bacteria efficiently managed to enhance the nodule parameters and leghemoglobin contents in horse gram plants in Cu-contaminated soils. Harmful effect of heavy metals on legume plants can be reduced by nodule endophytic bacteria (Kong et al. 2015).

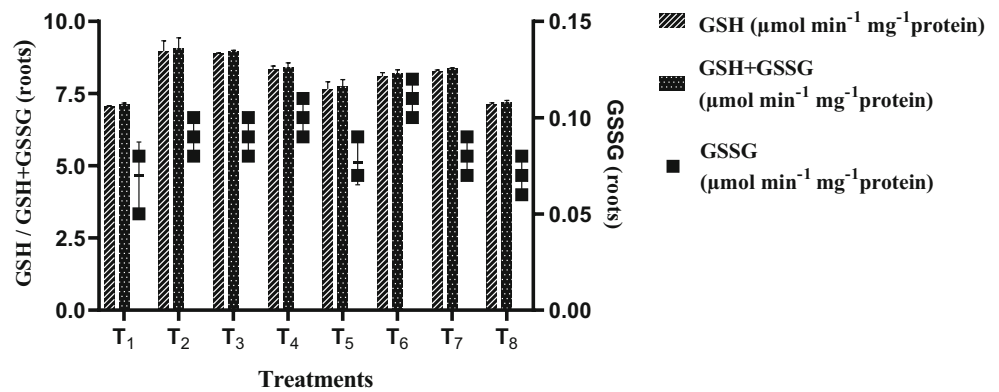
Plant growth-promoting microorganisms are preferably applied as soil amendment for improving soil fertility and crop growth (Mahmood et al. 2016). Seed bio-inoculation is

**Fig. 11** Effect of Cr(VI) and bio-inoculation on the contents (μmol min<sup>-1</sup> mg<sup>-1</sup> protein) of reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione (GSH + GSSG) in leaves of *M. uniflorum* var. Madhu at 60 days





**Fig. 12** Effect of Cr(VI) and bio-inoculation on the contents ( $\mu\text{mol min}^{-1} \text{mg}^{-1} \text{protein}$ ) of reduced glutathione (GSH), oxidized glutathione (GSSG), and total glutathione (GSH + GSSG) in roots of *M. uniflorum* var. Madhu at 60 days



another method but unsatisfactory survival of microorganisms is a major shortcoming owing to possible interaction with inhibitor compounds or with antagonistic microorganisms (Mahmood et al. 2016). In case of hydropriming, seeds are hydrated with water molecules to trigger metabolic activities in enhancing germination potential and adaptability to resist abiotic stress (Patra et al. 2019). Previous study has documented that *Rhizobium* bio-inoculation in integration with other beneficial bacterial strains showed promising outcomes with respect to crop growth and productivity (Wani et al. 2007b). Seed coating with microbial inoculum can be called as biopriming (Ashraf and Foolad 2005). Seed hydropriming subsequently followed by drying and biopriming activates metabolic activities; strengthens stress tolerance properties, viz, heavy metal and salt; and also enhances rate of germination (Anitha et al. 2013; Moeinzadeh et al. 2010; Patra et al. 2019). Consequently, in this investigation, seed coating of hydroprimed seeds emerge out to be as the preferably suitable method of application for nodule endophytes compared to that of non-primed seeds.

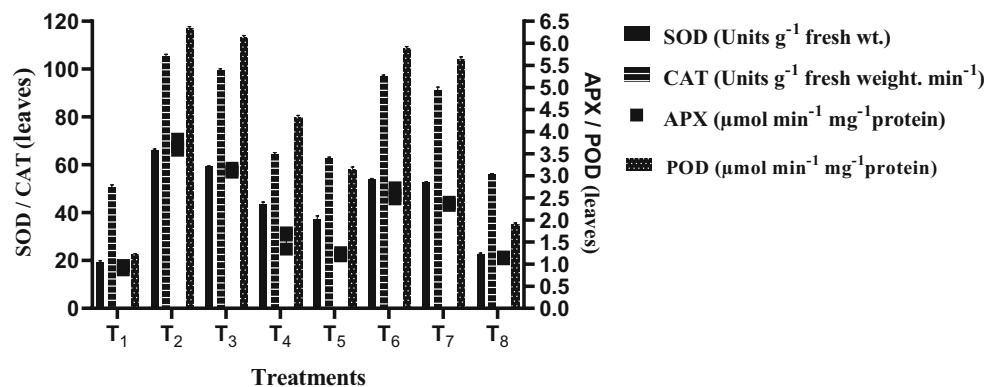
**Effect of Cr(VI)-tolerant nodule endophytes on biochemical constituents**

**Total chlorophyll, total soluble protein, reducing sugar, and total carbohydrate** The pots treated with combined inoculation of AS03 and AS05 strains showed encouraging results in

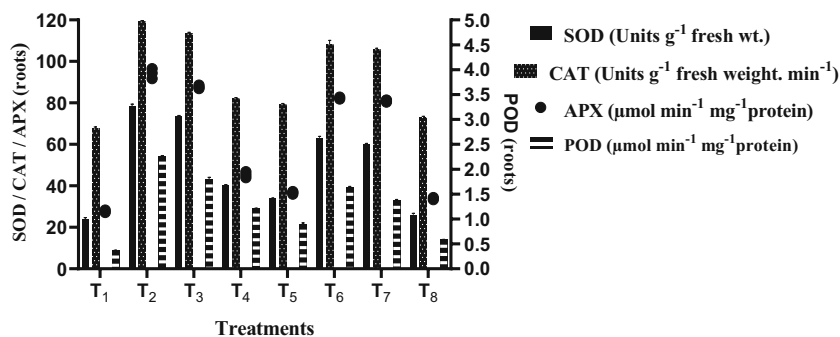
case of total chlorophyll, total soluble protein, reducing sugar, and total carbohydrate contents of leaves of *Macrotyloma uniflorum* at 60 days of growth. Combined inoculation of both the Cr-tolerant strains through seed inoculation with hydropriming showed better performances in comparison to non-primed seeds. However, sole inoculation of AS03 showed the lowest concentrations of total chlorophyll, total soluble protein, reducing sugar, and total carbohydrate contents in leaves (Fig. 6). Cr toxicity adversely affects total chlorophyll concentration, proteins, sugar and carbohydrates (Rai et al. 2014; Dhali et al. 2020; Sharma et al. 2020). Plants growing under Cr stress were assayed with declining biosynthesis of chlorophyll pigments (Sharma et al. 2019). Application of nodule bacteria could mitigate the harmful effect of Cr on chlorophyll, proteins, sugar, and carbohydrate contents of plant (Stambulska et al. 2018).

**Photosynthetic performances** Data presented in Fig. 7 represents photosynthetic behavior of the crop recorded after 60 days of growth period. Photosynthetic rate was higher in the control pots (T<sub>1</sub>) (no Cr toxicity) followed by pots applied with combined inoculation of AS03 and AS05 (T<sub>8</sub>). Cr toxicity in soil decreased the above all photosynthetic performances of the plant. However, plants treated with both AS03 and AS05 showed promising results in terms of photosynthetic behavior of plants, whereas sole inoculation of either of the bacteria could not benefit the crop in combating Cr

**Fig. 13** Effect of Cr(VI) and bio-inoculation on superoxide dismutase (SOD), catalase, (CAT), ascorbate peroxidase (APX), and guaiacol peroxidase (POD) in leaves of *M. uniflorum* var. Madhu at 60 days



**Fig. 14** Effect of Cr(VI) and bio-inoculation on superoxide dismutase (SOD), catalase, (CAT), ascorbate peroxidase (APX) and guaiacol peroxidase (POD) in roots of *M. uniflorum* var. Madhu at 60 days



stress. Cr stress impairs chlorophyll biosynthesis enzymes (Muslu and Ergün 2013). This can lead to deterioration of optimum photosynthetic behavior in leaves (Zlobin et al. 2015). Vernay et al. (2007) also reported decreasing trend in net photosynthetic rate of plants owing to Cr stress. We report here the synergistic effect of both the strains AS03 and AS05 could be able to enhance photosynthetic behavior in horse gram growing under Cr stress. In a recent study published by Kullu et al. (2020), arbuscular mycorrhizal fungi association with a grass crop *Brachiaria mutica* could be able to enhance the photosynthetic behavior under Cr stress.

**Flavonoids, proline, and total phenolic content** Flavonoids, proline, and total phenolic contents in the leaves of *Macrotyloma uniflorum* were estimated at 60 days (Fig. 8). Pots applied with AS03 through seed inoculation (T<sub>2</sub>) recorded the highest contents of flavonoids, proline, and total phenolics followed by the treatment T<sub>3</sub>. The lowest concentrations of flavonoids, proline, and total phenolics were noticed in the pots applied with combined inoculation of AS03 and AS05 (T<sub>8</sub>) which was at par with control pots (T<sub>1</sub>). However, bio-inoculation through solid carrier-based biofertilizer showed less concentrations of flavonoids, proline, and total phenolics compared to seed bio-inoculation. Flavonoids, proline, and total phenolic contents are the part of non-enzymatic antioxidative system which is secreted by plants owing to abiotic stress including chromium (Kasote et al. 2015; Patra et al. 2018; Kullu et al. 2020). The external application of nodule endophytes (AS03 and AS05) decreased the secretion

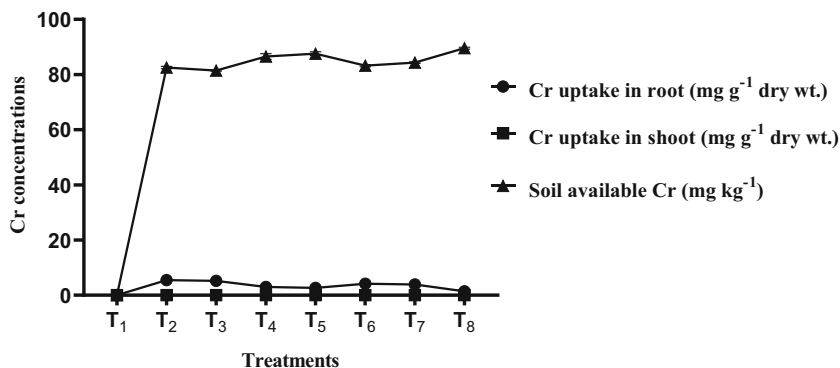
of non-enzymatic antioxidative systems in plants growing under Cr stress.

### Antioxidant enzymes and reactive oxygen species

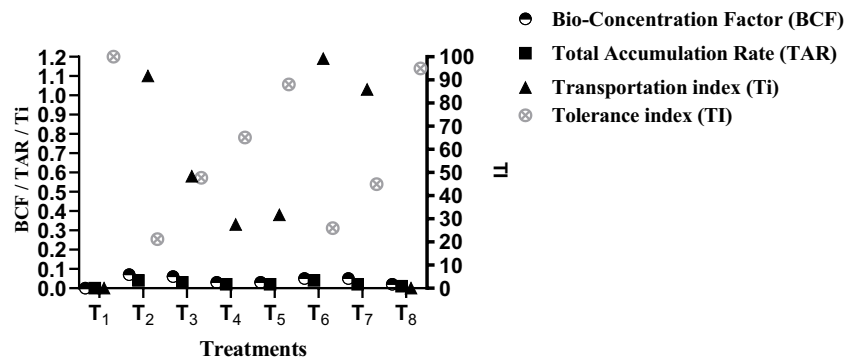
The contents of reactive oxygen species (H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>, OH<sup>-</sup>) and malondialdehyde (MDA) were significantly higher in treatment T<sub>2</sub> (seed bio-inoculation of AS03) compared to combined application of both the strains, but the data were at par with T<sub>3</sub> (seed bio-inoculation of AS05) (Figs. 9 and 10). Pots treated with T<sub>8</sub> appeared to have significantly lower concentrations of H<sub>2</sub>O<sub>2</sub>, O<sub>2</sub><sup>-</sup>, OH<sup>-</sup>, and MDA compared to T<sub>2</sub> and T<sub>3</sub> (sole applications of AS03 or AS05). Hydropriming and seed bio-inoculation with both the strains performed better compared to non-primed seeds.

Data regarding antioxidant enzymes, viz, GSH (reduced glutathione), GSSG (oxidized glutathione), and GSH + GSSG (total glutathione) contents in leaves and roots of *Macrotyloma uniflorum*, are recorded at 60 days of growth presented in Figs. 11 and 12. GSH and GSSG concentrations of leaves and roots showed greater variations. GSH and GSSG contents increased significantly with sole application of strain AS03 compared to combined application of AS03 and AS05. The lowest values of GSH + GSSG were obtained in the control pots which were at par with T<sub>8</sub> (combined application of AS03 and AS05). Additionally, SOD, CAT, APX, and POD values in leaves and roots were also observed at 60 days (Figs. 13 and 14). Combined application of both the Cr(VI)-tolerant endophytes AS03 and AS05 significantly influenced

**Fig. 15** Effect of Cr(VI) and bio-inoculation on soil avail Cr and total Cr contents in roots and shoots of *M. uniflorum* var. Madhu at 60 days



**Fig. 16** Effect of Cr(VI) and bio-inoculation on BCF, TAR, Ti, and TI of *M. uniflorum* var. Madhu at 60 days



the SOD, CAT, APX, and POD values compared to sole application of AS03 or AS05. The highest values in terms of SOD, CAT, APX, and POD were recorded in T<sub>2</sub> (AS03) followed by T<sub>3</sub> (AS05). Hydropriming of seeds with combined seed inoculation of AS03 and AS05 showed lower values of SOD, CAT, APX, and POD compared to non-primed seeds (T<sub>4</sub>) though it could not be able to influence significantly. Generation of ROS, malondialdehyde by crops, is definitely an indicator of stress (Shahzad et al. 2018; Adrees et al. 2015; Rout et al. 2019). Effective scavenging of ROS components accomplished by antioxidant defense system is also the crucial factor for maintenance of crop growth under Cr stress (Gill and Tuteja 2010). According to previous published studies, effective nitrogen fixing bacteria can diminish the toxic effect of Cr by minimizing ROS generation and modulating effective scavenging executed by both enzymatic and non-enzymatic antioxidant defense system (Stambulska et al. 2018). It is clear from the antioxidant enzymes and ROS data that the strain AS03 alleviated Cr(VI) stress but it is quite negligible to that of strain AS05 and the combination of both.

**Effect Cr(VI)-tolerant nodule endophytes on Cr (VI) bioaccumulation**

Data on chromium concentrations in soil, roots, and shoots showed variations (Fig. 15) at 60 days. No significant differences were observed in soil with respect to available Cr among all the treated plants at 60 days of crop growth. Roots were found with higher amount of accumulated chromium compared to shoots. It is noteworthy to express here that no chromium was found in shoots of *Macrotyloma uniflorum* in treatment T<sub>9</sub> (combined application AS03 and AS05). The treatment T<sub>5</sub> (hydropriming in combination with AS03 and AS05) also recorded very negligible amount of chromium in shoots. Combined application of both the Cr-tolerant strains AS03 and AS05 significantly influenced the plants to accumulate less chromium in roots and negligible in shoots.

BCF was very negligible, and TAR was quite negligible in plants treated with T<sub>8</sub> (Fig. 16). Ti recorded as zero in T<sub>8</sub> (Fig. 16). Ti for plants showed more than 90% in case of combined application of AS03 and AS05 (Fig. 16). *Bacillus aryabhatai* AS03 and *Rhizobium pusense* AS05 when applied as sole could not show concurring results to the plant, though they could minimize the chromium uptake in plants. Potential for Cr tolerance in case of bacteria is specific for a particular species and strain (Kong et al. 2015). In case of legume plants, both the plant and rhizobia compete for Cr accumulation, and rhizobia being more tolerant accumulates higher Cr in root nodules, ultimately minimizing its translocation to shoots (Stambulska et al. 2018). Previous studies also documented the bioremediation potential of plant growth-promoting bacteria against heavy metals in various leguminous plants (Cervantes et al. 2001; Stambulska et al. 2018). As the above findings suggested no Cr bioaccumulation in shoots at 60 days; hence, the edible part (seed) of the legume is safe for human consumption. Besides, our findings also recommend application of biochar as a carrier material for biofertilizer formulation.

**Conclusion**

Hexavalent chromium contamination has adverse effect on soil as well as agriculture and hence directly hampering the local farming community of Sukinda area, Jajpur District, Odisha, India. The present study is focused on the mitigation of challenges of Cr-toxic soil for healthier crop growth. From this study, it can be concluded that synergistic effect of native strains of Cr(VI)-tolerant nodule endophytes; *Bacillus aryabhatai* AS03 (MT645244) and *Rhizobium pusense* AS05 (MT645243) completely decreased Cr accumulation in shoots and enhanced morpho-physicochemical characteristics and tolerance indices of the plant. We established an approach to reduce the toxic effects of Cr(VI) on *M. uniflorum* by utilizing the two novel nodule endophyte strains which contributed towards better crop growth and benefited the nutritionally as well as medicinally important leguminous crop.

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**Author contribution** Shilpee Dhali: formal analysis, data curation, writing—original draft, investigation. Madhusmita Pradhan: formal analysis, data curation, writing—original draft and review. Ranjan Kumar Sahoo: writing—review and editing, methodology, statistical analysis. Santanu Mohanty: review and editing, data curation, conceptualization. Chinmay Pradhan: methodology, conceptualization, data curation, project administration, supervision.

**Data availability** Not applicable

## Declarations

**Ethics approval and consent to participate** Not applicable

**Consent for publication** Not applicable

**Conflict of interest** The authors declare no conflict of interest.

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