Abstract

In the current study, we aimed to elucidate the bioremediating potential of endophytic fungi during various heavy metals stresses to the cucumber (Cucumis sativus) plants. Preliminary screening showed that endophyte (Aureobasidium pullulans BSS6) produced IAA and extra-cellular enzymes (phosphatase, cellulase and glucosidase). Results showed that antioxidant activities (catalase, peroxidase and reduced glutathione) were significantly enhanced and lipid peroxidation inhibited during stress conditions in A. pullulans BSS6 inoculated plants, particularly in LTM stress. The ameliorative effects of BSS6 were evident from the quantification of results and were found to significantly regulate soil enzymatic activities: including (β-glucosidase, phosphatases and cellulases) during STM and LTM. Hence, endophytic BSS6 proved to be efficient in improving cucumber tolerance during metal contamination via regulating soil enzymatic activities to reduce metal uptake and boost the antioxidant system.

Introduction

Endophytic fungi can closely interact with host plants and establish symbiotic relationships by colonizing the inner tissues without harming the host. The association of plants and endophytes, particularly fungi, is considered of great significance to the tolerance of both biotic and abiotic stress (Khan et al. 2015). Fungal endophytes have been acknowledged as producing beneficial secondary metabolites which strengthen crop plant defense systems and alleviate various environmental stresses such as salinity, flooding, drought, temperature and heavy metal induced toxicity, etc. (Chadha et al. 2015; Azad and Kaminskyj 2016). The development of a symbiotic interaction between plants and fungal endophytes can also lead to significant enhancements in plant growth, yield and production, which are mainly attributed to fungal production of various plant growth promoting regulators such as indole-3-acetic acid and gibberellic acids, while in return plants provide shelter (Coleman-Derr and Tringe 2014; Xia et al. 2016; Hamayun et al. 2017). In addition to phytohormones, fungal endophytes have also been reported as a potential source of extracellular enzymes such as cellulases, protease, lipases, phosphatase, amylases, etc., which promote growth and influence soil enzymatic activities (Khan et al. 2016a; Ali et al. 2017a). Endophytic fungi with the potential to produce exoenzymes can be more beneficial to plant’s ability to manage, uptake, and translocate essential nutrients from the soil (Kotroczo et al. 2014; Hagmann et al. 2015). The abilities of microbes with an enhanced potential for exoenzyme production can also help the host plant to remediate external chemical stresses such as heavy metal exposure, because of the enzymes ability to form metal complexes (Ali et al. 2017b).

Heavy metals such as lead (Pb) cause adverse effects on crop species at the physiological, biochemical, molecular and cytological levels. Previous studies have shown that Pb is also capable of inhibiting shoot and root germination as described by Wang et al. (2011) and Lamhamdi et al. (2011) in wheat. Similarly, Cd has been reported to disrupt the enzymatic antioxidant system of plants by causing imbalances in cellular redox status via inducing oxidative stresses (Ali et al. 2014; Li et al. 2015). Plants adopt different biochemical and molecular strategies i.e hormonal regulation, antioxidative enzymes, vacuolar, transporters and metal chelators to counteract the adverse effects of metal contamination during metal pollution (Dubey et al. 2010; Hossain et al. 2010; Galano et al. 2015).

Therefore, it is necessary to improve phytoremediation strategies for low-biomass plants, especially crops, in order to mitigate the high level of metal contamination in the soil (Abhilash et al. 2016; Wood et al. 2016). For this purpose, endophytes have the ability to provide nutrients to host plants and confer beneficial strategies including sequestration, chelation, biotransformation, etc., for remediating heavy metal toxicity (Aly et al. 2011; Mohd et al. 2017). However, emphasis has been given to endophytic bacteria in exploring their bioremediating and growth enhancing potential (Gutiérrez-Ginés et al. 2014). Endophytic fungi, as a higher order species, are comparatively more compatible for bioremediating strategies due to higher biomass and the production of secondary metabolites under metal induced stress (Firmin et al. 2015; Khan et al. 2016b).
The sources of soil enzymatic activities have not been fully explored; however, the soil microbial community and plant life are considered major producers of these enzymes. Soil enzymes are reported to be involved in various biochemical reactions, detoxifying soil contamination, marinating soil texture and structure, conversion and decomposition of organic matter, etc. (Lestan et al. 2014; Ma et al. 2015a). Therefore, to assess the role of endophytic Aureobasidium pullulans BSS6 application on soil enzymatic activities under Pb and Cd contamination at two different time points i.e. after short term metal (STM) stress (24 h) and after long term metal (LTM) stress (7 days), different soil enzymatic analyses were deployed, which included β-glucosidase, phosphatases and cellulases. Recently Hassan et al. (2017) reported that endophytic Penicillium chrysogenum and Penicillium crustosum with indole-3-acetic acid (IAA) and extracellular enzyme (amylase, pectinase, xylanase, cellulase, CMCase) producing potential played a significant role in establishing a sustainable crop production system. Various fungal endophytes with IAA producing ability have been reported as promoting plant growth and remediating metal contamination (Khan et al. 2015; Khan et al. 2016b). Aureobasidium pullulans BSS6 was previously isolated from the stem of the frankincense tree (Boswellia sacra) and was found to actively produce extracellular enzymes such as glucosidase, phosphatases and cellulases, and IAA as well as promote plant growth and development (Khan et al. 2016b). The B. sacra plants are reported to be capable of overcoming extremely hostile environments, such as limited water and nutrients, the severity of extreme temperature, etc., (Khan et al. 2016a). However, less is known about the endophytic fungi isolated from B. sacra and their ability to promote plant growth under abiotic stress, including metal toxicity.

Moreover, a plethora of research studies have demonstrated that cucumber (Cucumis sativus) plants have developed a sophisticated system for the detoxification and tolerance of heavy metal toxicity. As Janicka-Russak et al. (2012) reported, cucumber plants activate plasma membrane heavy metal ATPase activity and heat shock proteins for survivability when encountering heavy metal toxicity, including cadmium and copper stress. Similarly, plasma membrane H+/Pb2+, H+/Mn2+ and H+/Ni2+ antiporters and secondary membrane stimulation are reported to provide significant tolerance to cucumber plants under heavy metal toxicity (Migocka and Klobus 2007; Jia-Wen et al. 2013). Keeping in view the metal resisting and tolerance potential of cucumbers, we aimed to investigate the symbiotic influence of phytohormones and enzymes produced by endophyte A. pullulans BSS6 for strengthening cucumber (C. sativus) tolerance to Pb/Cd toxicity by regulating stress related oxidative systems and influencing the soil enzymatic activities of metal contaminated soil. We also aimed to assess the influence of A. pullulans BSS6 inoculation on the enzymatic activities of metal contaminated soil and growth promoting abilities in cucumber plants in a time dependent manner.

**Material and methods**

**Fungal growth, survival in heavy metals and near-infrared spectroscopy (NIRS) analysis for IAA production**

Endophytic A. pullulans BSS6 was previously isolated from the stem of the frankincense tree (B. sacra) and identified through molecular methods, including genomic DNA extraction, PCR amplification of the internal transcribed spacer (ITS) region, and phylogenetic analysis for constructing a phylogenetic tree (Khan et al. 2016a). Based on these findings, endophytic BSS6 was identified as A. pullulans with accession number ‘KX233836.’ The endophytic strain was grown on potato dextrose agar (PDA) plates amended with 5 mM lead Citrate (C12H10O4Pb3) and 5 mM cadmium sulfate hydrate (3CdSO4.8H2O). The plates were kept in an incubator at 28°C for 8 days in darkness in order to assess the survival ability of BSS6 against different heavy metal stresses as described by Khan et al. (2016b). After 8 days of incubation, results showed that BSS6 showed marked growth in culture even in the presence of Pb and Cd toxicity. The fungal strain was grown in potato dextrose broth (PDB) for 14 days at 30°C in a shaking incubator (140 rpm) for further experiments on plant-microbe interaction. The BSS6 was previously found to actively produce IAA (3.11 ± 0.29 nmol/mL) as analyzed through UPLC-MS (Khan et al. 2016a). However, to further validate the results, near-infrared spectroscopy was used for the first time for this analysis. The A. pullulans BSS6 was centrifuged (10,000×g for 12 min at 4°C) and the supernatant was analyzed on NIR. The IAA standard (1.0 mM stock) was prepared in methanol with different concentrations and we performed the near-infrared spectra (NIRS) in triplicates. NIRS data was analyzed using partial least squares through the Unscrambler version 9.0.

**Interaction of endophytic BSS6 and Cucumis sativus under Pb and Cd stress**

Seeds of local Omani C. sativus were obtained from Natural and Medicinal Plant Research Center, University of Nizwa, Oman, and surface-sterilized by washing with 70% ethanol for 30 sec followed by 2.5% sodium hypochlorite for 30 min and several rinsing steps with autoclaved double distilled water. In order to investigate the plant-microbe interaction with Pb and Cd polluted soil, seeds were sown in autoclaved pots (12-cm diameter) holding 300 g of horticulture soil (pH 5–7, bulk density under 0.3 mg/m3 and EC ≤12) containing coco peat (68%), perlite (11%), zeolite (8%) and peat moss (35%) as well as micronutrients available as NH4NO3 (~0.09 mg g−1; P2O5 ~0.35 mg g−1; NO3−~0.205 mg g−1; and K2O ~0.1 mg g−1) and transferred to a growth chamber (day/night cycle: 14 h; 28°C/10 h; 24°C; relative humidity 60–70%; light intensity 1000 E m−2 s−1 Natrium lamps). BSS6 grown in PDB medium was centrifuged for harvesting fungal mycelia. The mycelia (3–5 g) and culture filtrate (20 ml) of BSS6 were added to their respective plants, whilst the control plants only received endophyte-free culture filtrate. The plants with and without endophytes were grown for 15 days. Then, 50 ml solutions containing a total of 144 and 78.4 mg per pot of either Pb (2.0 mM) or Cd (2.0 mM) respectively, were applied to the plant’s root zone every 24 h for 7 days to keep plants under stressed conditions. To avoid the leaching of metal, plants were irrigated with distilled water before metal treatment. After short term metal (STM) stress (24 h) one batch of plants was harvested to determine short metal stress effects, while after long-term (7 days) heavy metals stress (LTM), another batch of plants was harvested. The experimental design involved the following treatments: (i) control fungi free media treated plants without metal stress; (ii) endophytic fungal-treated plants without metal stress, (iii) metal-treated
plants (Pb and Cd); (iv) fungal-inoculated plants with metal stress. The experiment was performed in triplicate, with 15 replicates per treatment. After harvesting, photosynthetic pigments (chlorophyll a, b and total carotenoids) and plant lengths were determined as described by Lichtenenthaler (1987).

**Metals analysis in plants tissue and soil**

For measuring Cd and Pb uptake and determination of bioavailability, representative fresh plants as well as soil samples were randomly selected from each replicated treatment. Plants were carefully harvested and divided into roots and shoots. The roots were carefully washed with double-distilled water to remove all debris and apoplastic contents. The samples were acid digested and analyzed on inductively coupled plasma optical emission spectroscopy (ICP-OES, Varian Vista-PRO RL, Palo Alto, CA, USA) after digestion with concentrated HNO₃ according to the protocol described by Bilal et al. (2018a).

**Determination of antioxidant and related enzymes in inoculated and non-inoculated plants**

The extent of lipid peroxidation was determined according to Ohkawa et al. (1979) by using 0.2 ml 8.1% sodium dodecyl sulfate, 1.5 ml 20% acetic acid (pH 3.5), and 1.5 ml 0.81% thiobarbituric acid tissue homogenate extracted with 10 mM phosphate buffer (pH 7.0). Content of reduced glutathione according to Ellman (1959), as reported by Khan et al. (2017) and absorbance was determined at 412 nm, and the GSH concentration was calculated by a standard curve. Catalase activity was assayed by the method described by Durrant and Dong (2004), by using Tris–HCl buffer (pH 7.0) containing 3 mM MgCl₂, 1 mM EDTA, and 1.0% PVP. Catalase activity was estimated by the decrease in absorbance of H₂O₂ at 240 nm, and 1-U of catalase was defined as micrograms of H₂O₂ released per milligram protein per minute. Peroxidase (POD) activity was measured as described by Bilal et al. (2017) with some modification. The amount of purpurogallin formed was determined by the absorbance at 420 nm, only the use of H₂O₂ in polyphenoloxidase. One unit of peroxidase and polyphenol oxidase was defined as an increase of 0.1 U of absorbance.

**Soil extracellular enzymes under Pb and Cd toxicity**

To quantify extracellular enzymes (β-glucosidase, phosphatases, and cellulases), the method of Khan et al. (2016a) was adopted with some modifications. Briefly, the soil 1 g/50 mL acetate buffer (50 mM; pH 5.5) samples from all treatments were incubated (26°C) and shaken (150 rpm), with each sample replicated five times in Erlenmeyer flask (100 mL). After 24 hr, the clear supernatant was obtained from each flask using centrifugation (4°C, 12,000 rpm for 10 min). The known standard (4-methylumbelliferone) and respective florigenic substrate for each enzyme were prepared according to Khan et al. (2016a). A pre-optimized fluorescence spectrophotometer (Shimadzu, Tokyo, Japan) was used to read the absorbance with 360 nm excitation and 460 nm emission at time initial and 30-minute intervals for 2 h. The readings were calculated according to this formula: Activity (μM⁻¹min⁻¹mL⁻¹) = slope of concentration versus time in hours.

**Statistical analysis**

Experiments were independently performed in triplicate and the values obtained are presented as the means ± standard deviation (SD). Data obtained showing the effect of metal toxicity on the growth attributes of plants were subjected to t-test using online GraphPad Prism software to determine the significant difference among treatment means at P < 0.05. The biochemical analyses were performed with Three-way analysis of variance (ANOVA) using GraphPad Prism software (version 7.0, San Diego, CA, United States) followed by Bonferroni post hoc test with a P < 0.05.

**Results**

**Screening for production of IAA by BSS6 by Near-infrared spectroscopy (NIR)**

The different concentrations of IAA standard and the culture filtrate of BSS6 were subjected to NIR spectroscopy (wavenumber 10000–4000 cm⁻¹). The NIRS spectra of the data showed scattering effects due to reflection or absorbance of IAA (Figure 1). Prominent absorption peaks were between 5500–7000 cm⁻¹. To quantify the amount of IAA for A. pullulans BSS6 Partial Least Square regression (PLSR) models were built by using 70% of their standards as a training set at their optimum spectral treatment conditions. In this case, factor 1 contains 47% of the total variation in the data. In fact, the loading plot indicates each variables (wavenumbers) importance to predicting the property (IAA). The absorption peaks in regions from 3500 to 3700 cm⁻¹ are due to IAA. The PLSR analysis showed that the A. pullulans BSS6 produces 48.23 ± 2.12 μmol/50 mL of culture medium.

**Inoculation of BSS6 under metals stress improve plant growth and photosynthetic pigments**

Inoculation of endophytic BSS6 revealed significant symbiotic effects on host plant C. sativus growth under Pb and Cd stress at two different time points (STM and LTM stress). In assessing the growth promotion caused by BSS6 under short-term metal stress and LTM stress, we found that the length of the host plant was significantly enhanced (P < 0.05) in BSS6 infected plants as compared to non-inoculated plants in the absence of metal stress (Table 1 and Supp. Table 1). In the short-term condition, both Pb and Cd stress significantly reduced plant length in non-inoculated plants as compared with inoculated plants. Endophytic fungal BSS6 inoculated plants exhibited marked enhancement (18% and 20%) in total length and increased dry weight in STM and LTM stress. To further validate the symbiotic interaction of endophytic BSS6, photosynthetic pigments such as chlorophyll and total carotenoids were assessed. During stress conditions, BSS6 inoculated plants exhibited significantly (P < 0.05) higher content of chlorophyll a and chlorophyll b and carotenoids than non-inoculated in both STM and LTM treatment (Figure 2). However, under normal growth conditions i.e. in the absence of metal stress, both inoculated and non-inoculated plants of STM treatment exhibited similar total carotenoid content with no statistical significance.
The effect of endophytic BSS6 application on metal (Pb/Cd) accumulation was assessed in plants in order to describe the remediating role of fungal-host interaction. Neither Pb nor Cd accumulation was found in fungal treated as well as non-treated plants in STM and LTM treatment under control growth conditions. Under stress conditions, BSS6 inoculated plants presented a significantly ($p < 0.05$) reduced accumulation of Pb and Cd ions, as compared to non-inoculated plants both in STM stress treatment as well as LTM stress treatment (Figure 3 and Supp. Table 1). However, in STM as well as LTM stress treatment Cd ions were measured to have highly accumulated in fungal treated and non-treated plants. Results of the current study revealed that BSS6 infected plants accumulated approximately 24%, 52% and 53%, 46% lower Pb, Cd in STM treatment and LTM, respectively. These findings clearly suggest that association of BSS6 with host plants remarkably reduced metal accumulation and alleviated metal induced stress. The effect of endophytic BSS6 application on availability of Pb and Cd is displayed in Figure 3. In both stress time points, i.e. after STM and LTM stress, the application of BSS6 significantly reduced the availability of Pb and Cd in the soil. However, in the case of the STM stress time point, the amount of available metals in the soil was significantly higher as compared to the LTM stress point.
stress time point. However, BSS6 treated soil exhibited significantly ($p < 0.005$) lower amounts of Pb (51%, 39%) and Cd (23%, 35%) in both STM and LTM stress treatments, respectively than non-treated soil (Figure 3).

**Effect of BBS6 inoculation on mitigating oxidative stress in Cucumis sativus**

The current results revealed that the integrity of the functional membrane of the plant cell was severely influenced by metal toxicity. Higher levels of malondialdehyde (MDA) content are considered an indicator of induced lipid peroxidation in the functional membrane of the plant cell. In the current study, the interaction of endophytic BSS6 significantly reduced the rate of lipid peroxidation compared to non-inoculated plants as measured by a lower content of MDA in STM as well as LTM stress treatments (Figure 4). To further elucidate the oxidative stress mitigating role of BSS6 symbiosis with *C. sativus*, assessment of GSH content in plants was carried out. Under control condition, both in STM and LTM stress treatment, inoculated plants presented significantly ($p < 0.05$) higher amounts of GSH content as compared to non-inoculated plants (Figure 4 and Supp. Table 1). Similarly, in the case of Pb and Cd stress, significantly ($p < 0.05$) higher amounts of reduced glutathione were generated in fungal treated plants as compared to non-inoculated plants in STM and LTM stress treatment. However, inoculated and non-inoculated plants under LTM stress exhibited higher amounts of GSH content than both the inoculated and non-inoculated plants of STM stress treatment, respectively.

In the case of antioxidants enzymes, endophytic BSS6 association with the host significantly regulated CAT activity both under control conditions and metal stress conditions in STM as well as LTM stress treatment. However, BSS6 treated plants portrayed significantly higher levels of CAT activity in

![Figure 2. Effect of Pb and Cd stress on the chlorophyll and carotenoids contents of *C. sativus* plants inoculated with endophytic BSS6. For each set of treatment, the different letter indicates significant differences at $p \leq 0.05$ level based on the t-test.](image-url)
LTM stress treatment than non-inoculated plants, as well as displayed the highest levels of CAT measured, as compared to both inoculated and non-inoculated plants of STM stress treatment. In terms of POD activity, BSS6 treated plants exhibited significantly ($p < 0.05$) increased POD activity under control conditions as well as under metal stress.

Figure 3. Plant metal uptake and variations in bioavailable fraction of Pb and Cd in soil after endophytic BSS6 application to metal contaminated soil. Means with different letters are significantly different ($p < 0.05$).

Figure 4. Influence of BSS6 application on MDA and GSH concentration activity in host plant shoots at short-term metals stress time point and long-term metals stress time point under Pb and Cd contamination. Each value represents the mean ± standard error. Different letters on the bars represent significant differences between the treatments (Tukey’s HSD, $p < 0.05$).
conditions during STM and LTM stress treatment. POD activity with LTM stress treatment was detected to be markedly higher as compared to STM stress treatment (Figure 5). Under Pb and Cd stress, fungal inoculated plants of both STM and LTM stress treatment displayed approximately 21%, 32% and 25%, 18% greater POD activity than those of non-inoculated plants, respectively.

**Regulation of extracellular enzymes in the soil in response to STM and LTM stress**

Soil enzymatic activities were assessed in order to determine the quality and efficiency of soil that is damaged by anthropogenic activators. To evaluate the role of endophytic BSS6 application on soil enzymatic activities under Pb and Cd contamination for STM and LTM stress, different enzymatic analyses were deployed, which included β-glucosidase, phosphatases and cellulases (Table 2). In the absence of metal stress, endophytic BSS6 treated soil displayed significantly higher glucosidase and cellulase content, but not phosphatase, as compared to non-fungal treated soil in both STM and LTM stress treatments. In terms of metal contamination, Pb and Cd toxicity adversely affect soil enzymatic activities of non-fungal treated plants. In STM stress under Pb and Cd toxicity, fungal treated soil exhibited significantly higher (34%, 63%), (12%, 54%), (29%, 49%) higher levels of phosphatases, glucosidases and cellulases, respectively, as compared to non-treated soil. In LTM stress treatment, BSS6 treated soil under control conditions displayed significantly higher (p < 0.005) content of glucosidase and cellulase then non-treated soil. Moreover, the tenure of LTM stress adversely disturbed soil enzymatic activities by significantly reducing soil enzyme content. Application of endophytic BSS6 to soil under Pb and Cd stress significantly improved soil enzymatic activities as measured by significantly (p < 0.0005) higher phosphatase (36%, 39%), glucosidase (85%, 77%), cellulose (34%, 50%), respectively, than those of non-treated soil.

The soil samples from STM stress of all treated plants were subjected to NIR spectroscopy for the measurement of their absorption in the wavenumber range from 10000 to 4000 cm⁻¹ (Supp. Figures S1 and S2). It can be seen from their spectral data (Supp. Figures S1, S2, S3) that there is some scattering effect due to reflection and the graph is not

![Figure 5. Influence of BSS6 application on CAT and POD activity in host plant shoots at short-term metals stress time point and long-term metals stress time point under Pb and Cd contamination. Each value represents the mean ± standard error. Different letters on the bars represent significant differences between the treatments (Tukey's HSD, p < 0.05).](image)

![Table 2. Effect of BSS6 application on extracellular enzymes of metal treated soil.](table)

| Treatments            | Phosphatase (μM·min⁻¹·mL⁻¹) | Glucosidase (μM·min⁻¹·mL⁻¹) | Cellulases (μM·min⁻¹·mL⁻¹) |
|-----------------------|-----------------------------|----------------------------|---------------------------|
| **Short-term metal stress** |                             |                            |                           |
| Control               | 7.8 ± 1.04d                 | 3.2 ± 0.35b                | 52.16 ± 3.84c             |
| BSS6                  | 8.4 ± 1.05c                 | 4.9 ± 0.58a                | 64.96 ± 4.93b             |
| Lead (Pb)            | 3.5 ± 0.61e                 | 3.2 ± 0.38b                | 50.08 ± 4.13c             |
| Lead + BSS6          | 12.7 ± 1.13b                | 3.6 ± 0.61b                | 70.30 ± 5.05a             |
| Cadmium              | 4.9 ± 0.88e                 | 2.5 ± 0.26c                | 40.22 ± 3.27d             |
| Cadmium + BSS6       | 13.3 ± 1.22a                | 5.6 ± 0.87a                | 77.67 ± 5.21a             |
| **Long-term metal stress** |                             |                            |                           |
| Control               | 8.4 ± 1.38b                 | 2.7 ± 0.38d                | 29.83 ± 3.27c             |
| BSS6                  | 9.93 ± 0.63a                | 26.2 ± 1.58a               | 41.18 ± 3.95a             |
| Lead (Pb)            | 4.74 ± 0.71d                | 0.76 ± 0.08e               | 19.4 ± 2.73d              |
| Lead + BSS6          | 7.37 ± 1.04c                | 5.4 ± 1.24b                | 29.34 ± 4.28c             |
| Cadmium              | 4.36 ± 0.64d                | 0.85 ± 0.61e               | 18.63 ± 2.82d             |
| Cadmium + BSS6       | 7.06 ± 0.82c                | 3.7 ± 0.87c                | 37.93 ± 4.79b             |

Note: Soil enzymatic activities as influenced by BSS6 inoculation in Pb and Cd contaminated soil. Means with different letters are significantly different (p < 0.05).
very smooth. The spectra in figure S1 depicted that there are prominent absorption peaks in between wavenumber 4500–5500 cm\(^{-1}\). For LTM stress soil samples, the NIRS data spectra showed the same behavior, and this can be validated from their spectral data as given in supp. figures S4, S5 and S6. The spectra in Figures S4 and S5 implied that there are prominent absorption peaks in between wavenumber 45–5300 cm\(^{-1}\). The NIR data in both short-term and LTM stress showed that the BSS6 produce exoenzymes. These results showed that in LTM stress, the BSS6 produce more exoenzymes than in STM stress. Both the fluorescence spectrophotometer and NIR data showed that the BSS6 assist in the production of exoenzymes.

**Discussion**

Endophytic fungi with the ability to produce IAA, gibberellins and exoenzymes can improve the host-plant growth during normal and stress conditions (Khan and Lee 2013; Limtong et al. 2014). Our results from NIR validated our previous results of IAA production by *A. pullulans* BSS6. The current results also suggest that NIRS coupled with PLS regression analysis could be a more robust and authentic tool to analyze the IAA content in the culture filtrate of fungi. Our previous study by Khan et al. (2016a) revealed that BSS6 produced the highest amount of IAA and some other extra-cellular enzymes among *A. pullulans*, *Phoma medicaginis* and *Thielavia arenaria* endophytes. This potential can be of particular benefits to host plants during metal contamination, as previous studies have shown (Zhao et al. 2010; Corrêa et al. 2014). Extracellular enzymes can also mediate the utilization of organic sources of nitrogen and phosphorus in soil (Cairney and Burke 1998). This easy access to nutrients, in turn, helps the plant in increasing its biomass and overall growth. In the current study, we also found that the inoculation of *A. pullulans* BSS6 increased cucumber plant growth and photosynthetic potential with or without metal contamination. Previous reports showed that Pb/Cd toxicity negatively affects the photosynthetic process and related pigments in plants (Amooaghaie et al. 2017; Vera-Estrella et al. 2017). However, the endophytic symbiosis of fungus enhances the plant’s chlorophyll pigments (Yang et al. 2015; Yamaji et al. 2016). Similar results of enhanced chlorophyll and carotenoid contents were observed during the current study (Figure 3).

The improved growth of cucumber plants was also seen through the higher bioaccumulation of metal in the endophyte *A. pullulans* BSS6 inoculated plants as compared to control plants. The findings of the current study are in agreement with (Bilal et al. 2017; Zahoor et al. 2017) who found reduction of metal uptake in *Glycine max* L., and *Brassica campestris* L. by inoculating with endophytic *Paezicomyces formosus* LHL10, *Galactomyces geotrichum* WLL1, and *Mucor sp. MHR-7* under Ni, Zn, and Cr, Zn, Cu polluted soil, respectively. Reduction in the metal uptake by BSS6 inoculated plants might also be attributed to the locking down or absorption of toxic Pb and Cd in the vesicles or vacuoles of fungal mycelium, restricting their access to the host plant (Zahoor et al. 2017). The decrease in the Cd and Pb uptake by BSS6 inoculated plants might also be achieved by the immobilization of metals by endophytic BSS6 in soil. As the fungal chitin of cell walls and vesicles are also involved in the binding and storage of toxic metals which leads to a reduction in heavy metal uptake via inhibiting their translocation to the plants (Göhre and Paszkowski 2006; Lei et al. 2011). The reduction in available metals in the current study by BSS6 might be attributed to the biosorption and bioaccumulation capabilities of BSS6 in contaminated soil, as plant growth promoting endophytes have frequently been reported as bioremediating agents through either biosorption, bioaccumulation, and extracellular or intra cellular sequestration (Ma et al. 2016; Zahoor et al. 2017).

Metal toxicity in plants induces oxidative stress by the excess generation of reactive oxygen species (ROS), that adverse effect growth and yield. Therefore, in the current study, the antioxidant system of the *C. sativus* plant was evaluated under Pb and Cd stress condition. *A. pullulans* BSS6 inoculation remarkably influenced the physio-chemical apparatus of cucumber plants. The results show that the lipid peroxidation during STM stress was significantly lower in the case of Pb stress and higher in Cd stress as compared to LTM. However, inoculation of endophytic BSS6 significantly reduced the rate of lipid peroxidation in both short-term and LTM stresses compared to non-inoculated plants. The increase in the malondialdehyde of LTM as compared to short-term might be due to long exposure to metal toxicity, as increased exposure to metal toxicity led to depletion or inhibition of enzymatic activities in plants (Fernández-Fuegoa et al. 2017). The lower lipid peroxidation rate of BSS6 inoculated plants in the current study is consistent with (Khan et al. 2014; Merlos et al. 2016), who found a decrease in lipid peroxidation of endophytic *Penicillium janthinellum*, *AM Rhizophagus irregularis*, and *Leptodontidium* inoculated *Solanum lycopersicum*, maize genotypes and *Populus tremula x alba* under Cd, Cu and trace element polluted soil, respectively. Higher MDA levels are generated under heavy metal toxicity, indicating the impaired activities of lipid membrane related enzymes and leading to the loss of lipid membrane integrity (Kamran et al. 2015; Ma et al. 2016). The lower amount of superoxide anion and lipid peroxidation also showed that the endophytic BSS6 interaction assisted *C. sativus* in the reduction of membrane damage and oxidative stress under metal stress as reported from White and Torres (2010) and Bilal et al. (2018b). Furthermore, recruiting higher amount of GSH is an essential step in countering oxidative stress and the current findings revealed significantly (*p < 0.005*) upregulated GSH levels in the host after BSS6 inoculation at both STM and LTM stress time point under both Pb and Cd contamination as compared to non-inoculated plants. GSH has also been reported in the scavenging and detoxification of toxic by-products of lipid peroxidation (Yin et al. 2017) and also acts as precursor for the production of metal chelating phytochelatins, thus protecting plant macromolecules from metal-induced oxidative injuries. In the current study, the higher GSH content might be correlated with the lower accumulation of lipid peroxidation in inoculated plants which clearly signifies a defensive role for endophytic BSS6 in Pb and Cd-induced stress. Previously Bilal et al. 2017 reported similar findings of reduced lipid peroxidation and higher GSH accumulation by inoculating *S. lycopersicum* and *G. max* L. with endophytic *P. formosus* and *Penicillium janthinellum* under Ni and Al stress. Similarly, increases in the catalase (CAT) and peroxidase (POD) of BSS6 treated plants under Pb and Cd toxicity were detected. Previously, various studies have indicated that metal toxicity exhibits adverse effects on POD and CAT by
suppressing their activities (Tan et al. 2015; Wang et al. 2016). The activities of these enzymes are considered essential for the dismutation of $\text{H}_2\text{O}_2$ to $\text{H}_2\text{O}$ and molecular oxygen in the cells. Our results are in accordance with Jang et al. (2016), Mendarte-Alquisira et al. (2017) and Khan et al. (2016b), who reported remarkable enhancement in the POD and CAT activities of AM Glomus versiforme (Gv) inoculated Lonicera japonica, endophytic A. alternata RSF-6L inoculated S. nigrum and endophytic Lewia sp. inoculated Festuca arundinacea plants grown in Cd, hydrocarbons amended soil, respectively. The enhancement in the CAT and POD activities in endophytic BSS6 treated plants under metal toxicity indicates that inoculated fungi may regulate and activate genes responsible for encoding antioxidant enzymes and subsequently alleviate heavy metal induced oxidative stress (Wang et al. 2016).

Besides regulating plant’s physio-chemical mechanisms, endophytic fungal inoculation also interferes with the host enzymatic flux during nutrient uptake and translocation. We observed that Pb and Cd toxicity markedly lowered the rate of soil enzymatic activities. Previously, various studies have indicated that heavy metal contamination markedly inhibits soil enzyme potential by amalgamating with the enzyme–substrate complex and subsequently leading to denaturation of enzymatic protein (Ma et al. 2015a). Rhizospheric microbes with the ability to produce extracellular enzymes have been acknowledged for positively effecting both soil properties and plant tolerance during heavy metal contamination (Ali et al. 2017b). Soil enzymes are reported to be involved in various biochemical reactions, detoxifying soil contamination, marinating soil texture and structure, and conversion and decomposition of organic matter. In the present study, the application of endophytic BSS6 significantly mitigated the inhibitory effects of Pb and Cd toxicity by enhancing soil enzymatic activities. BSS6 inoculation remarkably improved soil phosphatase content in both STM as well as LTM stress treatment. An increase in the phosphatase activity of soil under metal contamination is one the mechanisms employed in avoiding and detoxifying heavy metals, as well as boosting plants tolerance to metals stresses (Azcón and Barea 1997). Thus, the enhancement of phosphatase activity in the soil strongly justifies BSS6’s role in metal detoxification as well as in promotion of plant growth. Our findings are in accordance with Ali et al. (2017a) and Curaqueo et al. (2014) who found significant improvement in the phosphatase activity of various heavy metal polluted soils. In the case of glucosidase activity, the finding of the current study was consistent with the phosphatase activity. Under Pb and Cd toxicity, significantly more glucosidase was detected in BSS6 non-treated soil as compared to BSS6 treated soil. The increase in the glucosidase activity of BSS6 treated soil in the current study is in accordance with the findings of Su et al. 2017 and Ali et al. 2017b who reported the enhancement of glucosidase activity of soil affected from Zn/Pb and As toxicity by inoculating with Streptomyces pactum and Trichoderma asperellum. Previously, various studies have shown similar trends of reduction in glucosidase activity in metal polluted soils and declared it highly sensitive to metal toxicity (Parelho et al. 2016). Therefore, keeping in view the current results, BSS6’s ability to maintain the glucosidase activity of soil under severe metal toxicity could be a potential means to support the soil microbial community and provide a friendly ecosystem which will ultimately improve plant growth and yield. Similarly, cellulase activity of the soil was severely inhibited by Pb and Cd toxicity in LTM stress treatment. However, BSS6 inoculation significantly enhanced the cellulase activity of Pb and Cd contaminated soil, compared to non-inoculated soil. Previously, the reduction in soil cellulase activity by metal contamination has also been reported by many studies (Jia et al. 2015; Xian et al. 2015). The improved cellulase activity of the soil not only boosts the conversion of carbohydrates and decomposition of organic matter, but is also considered essential for facilitating vertical spreading and colonization of endophytes in host plants (Ma et al. 2015b).

To the best of our knowledge, the present study demonstrates for the first time the Pb and Cd resistance and remediating mechanisms of endophytic BSS6. The phytohormone (IAA) producing capability of BSS6 was analyzed by near-infrared spectroscopy (NIRS), which revealed a significant production of IAA. This ability of BSS6 was correlated with significant growth enhancement of the cucumber in Pb and Cd toxic soil. BSS6’s association with the cucumber triggered plant growth and metal translocation in the tissue through modulation of the antioxidant system and regulation of soil extracellular enzymes. These results indicate that endophytic BSS6 is a promising phytoremediating agent for crops grown in Pb and Cd polluted soil and may be mixed with other soil fertilizing amendments. However, further molecular and transcriptomic studies are needed to provide an in-depth understanding of the underlying mechanism of plant-BSS6 interactions.

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